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REGULATION OF THE LIPID RAFT LOCALIZATION OF THE GAL/GALNAC LECTIN, AN ADHESIN ON THE SURFACE OF THE HUMAN PROTOZOAN PARASITE, ENTAMOEBA HISTOLYTICA

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REGULATION OF THE LIPID RAFT LOCALIZATION OF THE GAL/GALNAC LECTIN, AN ADHESIN ON THE SURFACE OF THE HUMAN PROTOZOAN PARASITE, *ENTAMOEBA HISTOLYTICA*

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ABSTRACT

Lipid rafts, sterol- and sphingolipid-rich membrane microdomains, have been shown to control virulence in a variety of parasites including *Entamoeba histolytica*, an intestinal parasite that causes dysentery and liver abscess. Parasite cell surface receptors, such as the Gal/GalNAc lectin, facilitate attachment to host cells and extracellular matrix. The Gal/GalNAc lectin binds to galactose or N-acetylgalactosamine residues on host components, and is composed of heavy (Hgl), intermediate (Igl), and light (Lgl) subunits. Although Igl is constitutively localized to lipid rafts, Hgl and Lgl transiently associate with this compartment in a cholesterol-dependent fashion. Exposure to *bonafide* Gal/GalNAc lectin ligands is associated with enrichment of the subunits in rafts. Direct lectin-ligand interactions and sufficient levels of both PIP_2 and calcium were shown to be necessary for lectin enrichment in rafts. Additionally, an initial analysis of both post-translational modifications and protein interactions that regulate the association of the lectin subunits with rafts was performed. Glycosylation, palmitoylation, and GPI-anchoring were all shown to have possible roles in regulating the localization of the lectin subunits. Depolymerization of actin was shown to not affect the localization of any of the three subunits; however, another cytoskeletal protein, α-actinin was shown to be a potential regulator of the localization of Hgl.

DEDICATION

To my parents, William and Joyce Goldston, and my husband Kirk Johnson, for all of your support and guidance. Mom and Dad, thanks for making education my number one priority from a very young age (even if I hated doing homework over the summer). Kirk, thanks for being there for me, and supporting whatever decisions I have made. I really couldn't have done this without your love and support.

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CHAPTER ONE

LITERATURE REVIEW

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Abstract

Lipid rafts, sterol- and sphingolipid-rich membrane microdomains, have been extensively studied in mammalian cells. Recently, lipid rafts have been shown to control virulence in a variety of parasites including *Entamoeba histolytica*, *Giardia intestinalis*, *Leishmania* spp., *Plasmodium* spp., *Toxoplasma gondii*, and *Trypanosoma* spp. Parasite rafts regulate adhesion to host and invasion, and parasite adhesion molecules often localize to rafts. Parasite rafts also control vesicle trafficking, motility, and cell signaling. Parasites disrupt host cell rafts; the dysregulation of host membrane function facilitates the establishment of infection and evasion of the host immune system. Discerning the mechanism by which lipid rafts regulate parasite pathogenesis is essential to our

understanding of virulence. Such insight may guide the development of new drugs for disease management.

Lipid rafts: afloat in parasite membranes

Lipid rafts are tightly packed, cholesterol- and sphingolipid-rich, membrane microdomains, which serve as a platform where protein-protein or protein-lipid interactions occur (56). Lipid rafts can be extracted from the membrane through the use of cold non-ionic detergents; therefore, detergent-resistant membrane (DRM) is often considered representative of lipid raft populations. Although these terms are related, they are not necessarily interchangeable. Therefore, in this review, we selected terminology that best represented the purification method utilized in the original studies. Rafts play roles in signaling pathways regulating a number of cellular processes including adhesion, motility, secretion, and invasion. The function of rafts often depends on the proteins found within these domains and raft-association of these proteins is determined, in part, by the presence of post-translational modifications (Box 1). Rafts have been identified in a number of protozoan parasites including *Entamoeba histolytica*, *Giardia intestinalis*, *Leishmania* spp., *Plasmodium* spp., *Toxoplasma gondii*, and *Trypanosoma* spp., (Table 1).

Table 1. Overview of cholesterol-rich membrane microdomain functions.

Parasite	Functions[a]
Entamoeba histolytica	▪ Facilitate attachment to host collagen and host cells ▪ Regulate fluid phase endocytosis ▪ Adhesion Molecules: Gal/GalNAc lectin localized to rafts in a PIP_2- and calcium-dependent manner
Giardia intestinalis	▪ Facilitate attachment to host intestinal cells
Leishmania spp.	▪ Facilitate attachment to, entry into, and replication within host macrophages ▪ Adhesion Molecules: GP63 family of parasite adhesion molecules is localized to rafts ▪ Regulate motility (flagellar proteins localize to lipid rafts) ▪ Host Rafts: Parasite protein, GP63, enters host rafts and cleaves host phosphatases which are important in IFN-ϒ signaling. Infection results in mislocalization of host raft proteins, CD1d and CD40, which leads to alterations in IL12- and IL10-based signaling
Plasmodium spp.	▪ Facilitate attachment to and invasion of erythrocytes ▪ May control protein sorting in rhoptries (rhoptry proteins, such as Pf34 and RAMA, localize to DRM) ▪ Regulate motility (glideosome protein complexes localize to DRM) ▪ Host Rafts: Composition of host rafts is altered during invasion
Toxoplasma gondii	▪ Regulate motility (glideosome protein complexes localize to DRM)
Trypanosoma brucei	▪ May regulate calcium signaling (proteins involved in calcium signaling, such as calflagin Tb24, localize to lipid rafts)
Trypanosoma cruzi	▪ Facilitate invasion of host cells by trypomastigotes, but not amastigotes ▪ Regulate receptor-mediated endocytosis of transferrin ▪ Control flagellar signaling ▪ May regulate calcium signaling (proteins involved in calcium signaling, such as PI-PLC and FCaBP, localize to lipid rafts) ▪ Host rafts: Host rafts are required for stage-specific adhesion, internalization, and intracellular survival of the parasite

[a]Information regarding the importance of lipid rafts in virulence is referenced throughout the text.

Protozoan parasites with an intracellular life cycle stage, such as *Leishmania* spp., *Plasmodium* spp., and *Trypanosoma cruzi*, have also developed a number of mechanisms to manipulate host cell lipid rafts for invasion, colonization, and immune system evasion. In addition to traditional lipid rafts, host cell caveolae, which are a specialized type of lipid raft containing caveolin proteins, are also manipulated by intracellular pathogens. Parasites utilize lipid rafts during multiple life cycle stages. In *E. histolytica* and *G. intestinalis*, for example, rafts mediate initial attachment to the host epithelial layer. DRM associated proteins in *Plasmodium* spp. are required for invasion of red blood cells (RBCs). *Leishmania* spp. manipulate signaling pathways emanating from host lipid rafts to evade the immune system. The reliance on lipid rafts for survival truly makes these membrane domains a 'life raft' for parasites; the use of these life rafts determines the success of parasitic infections, and whether the parasites will ―sink or swim.

Adhesion: anchoring parasites to hosts

Adhesion to host cells by parasites is an essential first step in the invasion process, and may be mediated by parasite lipid rafts. During infection, intestinal pathogens such as *G. intestinalis* and *E. histolytica* attach to host epithelial cells of the intestinal tract. Exposure of *G. intestinalis* to methyl-beta-cyclodextrin (MβCD), a cyclic compound that chelates cholesterol and disrupts lipid raft domains, abolishes its ability to adhere to Caco-2/TC7 cells (27). Parasite adhesion to host is unaffected when Caco-2/TC7 cells are exposed to MβCD, demonstrating that the lipid rafts of *G. intestinalis*, and not those of host cells, regulate adhesion (27). Exposure of *E. histolytica* to MβCD

reduces adhesion to host cell targets, such as collagen (35) and Chinese hamster ovary (CHO) cells (65). Thus, parasite lipid rafts appear to regulate binding of intestinal parasites to host. The role of host cell rafts in *E. histolytica* infection has not been investigated to date.

The role of rafts in attachment to host is further illustrated by the existence of parasite adhesion proteins within raft domains. One class of adhesion molecules is the GP63 family of zinc-dependent metalloproteases in *Leishmania* spp. (14). GP63, which is glycophosphatidylinositol (GPI)-anchored, is localized to lipid rafts of *Leishmania amazonensis* (14). In *E. histolytica*, the best characterized of the cell surface adhesion molecules is the galactose N-acetylgalactosamine (Gal/GalNAc) lectin) which is comprised of heavy (Hgl), light (Lgl), and intermediate (Igl) subunits. This protein complex binds to galactose and N-acetylgalactosamine residues on host components. Igl, which is GPI-anchored, is constitutively localized to rafts. Hgl, a transmembrane protein, and Lgl, a GPI-anchored protein, form a covalent dimer that is only transiently associated with rafts. For example, physical interaction between *E. histolytica* and Gal/GalNAc ligands on RBCs or collagen (24) leads to the enrichment of Hgl-Lgl dimers in rafts and, thus, co-localization of all three subunits. Cholesterol-loading of the membrane similarly enhances the enrichment of the Hgl-Lgl dimer in rafts (65). Interestingly, co-localization of these subunits in lipid rafts during cholesterol loading correlates with increased adhesion to CHO cells (65). Therefore, in *E. histolytica*, rafts may regulate the assembly and function of adhesion molecules.

In addition to adhesion, lipid rafts mediate the invasion process of a number of pathogens with intracellular life cycle stages. For example, invasion is inhibited in MβCD-treated trypomastigotes, but not MβCD-treated amastigotes of *T. cruzi* (19). This suggests the involvement of rafts in infection is stage-specific in *T. cruzi* (19). Infection by *Leishmania viannia braziliensis* involves attachment to and then subsequent phagocytosis by macrophages. MβCD exposure of the parasite reduces the infection rate of this pathogen in macrophages (67).

Parasite armament: DRM-associated rhoptry and surface proteins open the gangway for *Plasmodium* spp.

P. falciparum proteins that function in invasion of RBCs are localized to the DRM of *P. falciparum*. Many of these DRM-associated proteins were identified in proteomic analyses, an overview of which can be found in Table 2. Proteins that comprise the rhoptry, an organelle which is responsible for secreting proteins into host cytoplasm, were identified among the DRM associated proteins (Figure 1). Detergent-resistant proteins were found in both the rhoptry bulb and rhoptry neck. For example, Pf34, a rhoptry neck protein, and rhoptry associated membrane antigen (RAMA), a rhoptry bulb marker, are present in the DRM proteome of *P. falciparum* (45, 53). The localization of RAMA to DRM domains is necessary for proper trafficking of other proteins, such as the rhoptry associated protein (RAP) family, to the correct rhoptry compartment for secretion (47). Pf34 is proposed to be an adhesin that functions during invasion of erythrocytes (3). Pv34, the *Plasmodium vivax* homolog of Pf34, is also DRM-

associated, although its exact localization, rhoptry bulb or neck, is currently unknown (37). Rhoptry neck protein 1 (RON1), which is conserved across Apicomplexa, is a DRM-associated protein expressed in the schizont stage of *P. vivax* (39). Understanding the trafficking and the subcellular localization of rhoptry proteins is important as they are predicted to be involved in RBC invasion.

Surface proteins that are raft-associated are also important in invasion by *Plasmodium* spp. (Figure 1), and several are potential vaccine targets. GPI-anchored merozoite surface proteins, including MSP-1, MSP-2, and MSP-4, were all identified in the DRM proteome (53). In addition to its rhoptry localization, detergent-resistant Pf34 is also found on the surface of the parasite (2, 36, 53). Three detergent-resistant proteins containing Cys6 domains, Pf38/Pv38, Pf41/Pv41, and Pf12/Pv12, are present in *P. falciparum* and *P. vivax*, respectively. Pf38 is a GPI-anchored protein in *P. falciparum*, and is localized to the merozoite surface as well as to the rhoptries (53). *P. falciparum* Pf12 is localized to the merozoite surface, but its homolog, Pv12, is localized to the rhoptry neck in *P. vivax* (32). These Cys6 proteins are strongly recognized by antibodies of malaria-infected individuals (36, 53). Also, exogenous addition of these proteins moderately inhibited merozoite invasion (21), supporting their role in virulence. Together, these data support the potential of detergent-resistant surface proteins as vaccine targets

Table 2. DRM proteome of *Plasmodium* spp. and vector.

Protein Categories	*P. falciparum*[a, b]	*A. gambiae* midgut[c, d]	*P. berghei*[e, f]	*P. berghei* host protein[e]
Chaperones			•	
Cytoskeletal proteins	•			•
Formation of parasitophorous vacuole	•			
Glycosyl hydrolases		•		
GPI-anchored proteins	•	•		
GPI-binding proteins	•			
Immunoglobulin-like proteins		•		
Inner Membrane Complex	•			
Known ookinete interacting proteins[g]		•		
Lectins/receptors		•		
Membrane fusion events			•	
Merozoite surface proteins	•			
Multimembrane spanning proteins	•			
Multidrug resistance	•			
Peptidases		•		
Protease inhibitors		•		
Protein complex assembly			•	
Protein folding	•			•
Protein sorting			•	•
Proteins involved in adhesion/ invasion	•	•	•	
Rhoptry associated proteins	•			
Trafficking			•	•
Transporters		•		
Variant antigen superfamily			•	

[a]from (53).
[b]from (52)
[c]from (42)
[d]*A. gambiae* is the insect vector of *Plasmodium* spp.
[e]from (16)
[f]*P. berghei* is a closely related species to *P. falciparum* which infects rodents
[g]6 known ookinete interacting proteins (aminopeptidase N, 3 annexin like proteins, carboxypeptidase B, and scavenger receptor, croquemort homologue) were identified in the DRM proteome of *A. gambiae* and are candidates for use in transmission blocking vaccines

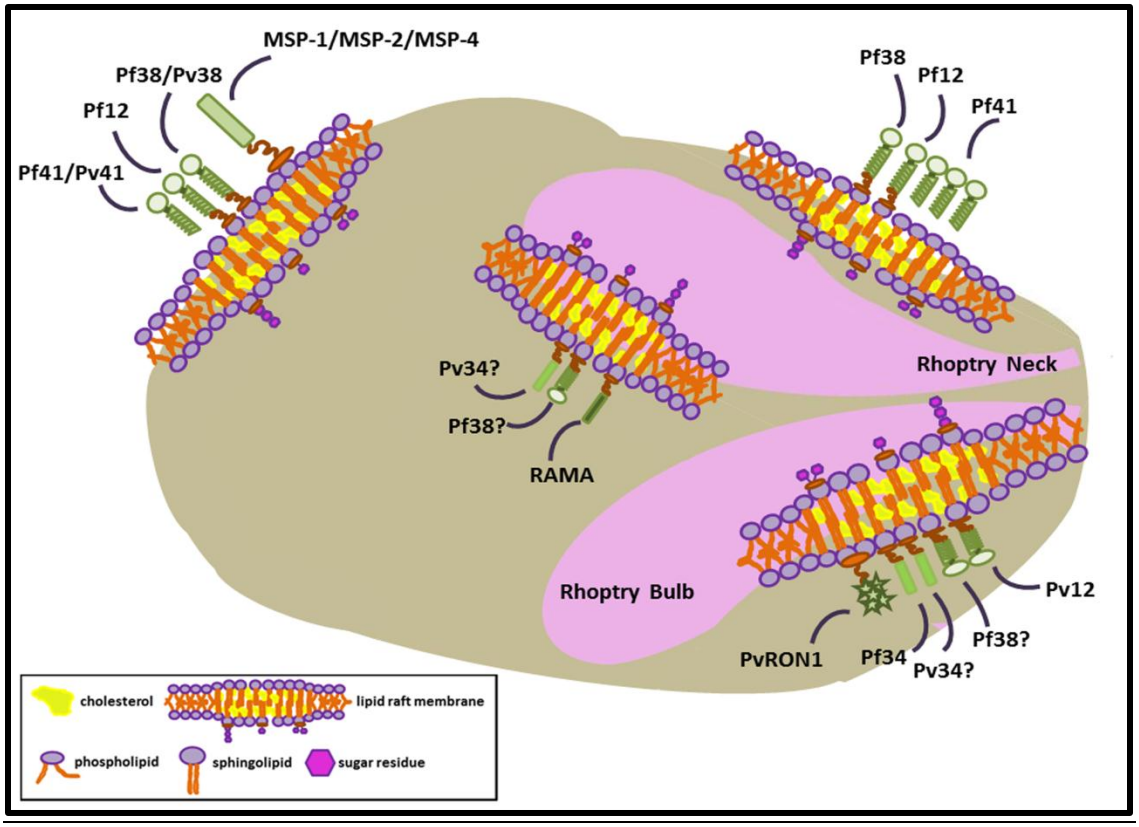

Figure 1. Proteins associated with DRM in *Plasmodium*. Several studies have identified detergent-resistant membrane associated proteins in *Plasmodium*. DRM proteins are commonly found on the merozoite surface and within rhoptries. MSP-1, MSP-2, and MSP-4 are GPI anchored surface proteins identified in studies using *P. falciparum* (53). Although illustrated at the basal end, these proteins are likely distributed in DRMs throughout the parasite surface (53). Additionally, a group of 3 proteins containing Cys6 domains, Pf41, Pf12, and Pf38, were identified in the DRM; Pf12 and Pf38 are likely GPI-anchored, while Pf41 has no membrane anchor, and may be complexed with other GPI-anchored proteins, such as Pf12 and Pf38 (2, 36, 53). Though Pf12 and Pf38 were classified as 'surface proteins', the location of Pf41 was more precisely determined. The majority of Pf41 was concentrated at the apical end of merozoite stage parasites (36, 53). Localization studies suggest that Pf38 is also localized to rhoptries, although the precise location within the rhoptry was not determined (53). Some DRM-associated proteins were localized to rhoptries, including RAMA, a known rhoptry bulb protein, and Pf34, Pv12, and PvRON1, known rhoptry neck proteins (32, 37, 39). Interestingly, Pv12 and Pf12 are found in different DRM locations in their respective *Plasmodium* species. Pf38 is localized to the merozoite surface in addition to rhoptries, though its precise localization within rhoptries has not been determined (36). Pv34, the *P. vivax* homolog of Pf34, is also localized to rhoptries, though it is uncertain whether it is associated with the bulb or neck region (37).

Endocytosis: taking on nutrients

Endocytic processes are important for parasite nutrient uptake, and thus, parasite growth and survival. Both non-specific and receptor-mediated endocytosis are mediated by vesicle trafficking, and studies suggest that vesicle trafficking in parasites relies on lipid rafts. For example, in *E. histolytica*, raft disruption by MβCD inhibits fluid phase endocytosis, a nonspecific vesicle trafficking event (31). Additionally, regions of the plasma membrane where receptor-mediated uptake of transferrin occurs in *T. cruzi* co-localize with a lipid raft stain, cholera B toxin, and a raft marker, flotillin-1, suggesting that transferrin uptake occurs in lipid raft regions (11). In the presence of MβCD or filipin, two raft-disrupting agents, transferrin uptake was inhibited in *T. cruzi* (10). Together, these data highlight the importance of parasite lipid rafts for survival; interestingly, host cell endocytic pathways are manipulated by intracellular parasites during the invasion process. Several examples of such host cell 'hijacking' can be found in 'Host cell lipid rafts: more than just docks' and 'Maintaining stowaway status: avoiding phagolysosomal acidification' sections.

Parasite motility: full steam ahead

The role of lipid rafts in motility has been established for a number of protozoan parasites. Apicomplexan parasites employ a glideosome, which contains the molecular machinery needed for motility (12). The glideosome of *Toxoplasma gondii* contains two myosin proteins, myosin A heavy chain and myosin light chain. It also contains two glideosome associated proteins (GAPs), GAP45 and GAP50, both of which anchor the

glideosome to the inner membrane complex (IMC) (12). In *T. gondii*, the glideosome is first assembled as a soluble complex containing myosin A heavy chain, myosin light chain 1, and GAP45 (12). This complex then becomes associated with cholesterol-rich DRM domains of the IMC, and this association can be disrupted by MβCD (12, 28). Glideosomes also regulate motility in *P. falciparum*. The *P. falciparum* homologs of *T. gondii* IMC proteins PfGAP50, PfGAP45, and myosin A, and two additional glideosome-associated proteins, PfGAPM1 and PfGAPM2, are also detergent-resistant (52, 53). Therefore, it is clear that lipid rafts are involved in apicomplexan glideosome-mediated motility.

Lipid rafts, and their associated proteins, are enriched in the flagellar membrane of kinetoplasts and also participate in cellular motility (12, 23, 61). In *Leishmania major*, small myristoylated protein 1 (SMP-1), a small dually acylated protein, is targeted to lipid rafts in the flagellum (59, 60). Another *L. major* SMP protein, SMP-2, has been identified, which is non-raft associated (23). Double knockout parasites, with loss of SMP-1 and SMP-2, exhibited shortened flagella and motility defects (59). Although the phenotype of SMP-1 single knockouts has not been discussed in the literature, the phenotype of the double knockout is rescued by re-introduction of DRM-associated SMP-1, but not SMP-2 (59). This demonstrates that DRM associated proteins, such as SMP-1, are critical for flagellar function.

Cell signaling: aye, aye, captain

The connection between lipid rafts and temporal and spatial regulation of cell signaling is well-established. Cell signaling in the parasite may result in changes within the parasite or within the host. For example, in *E. histolytica*, sufficient levels of phosphatidylinositol (4,5) bisphosphate (PIP_2) and intracellular calcium, which are important signaling molecules, are required for Gal/GalNAc lectin localization in lipid rafts (24). In *T. cruzi*, phosphatidylinositol specific phospholipase C (PI-PLC) resides in flagellar lipid rafts (13). PI-PLC hydrolyzes PIP_2 into inositol trisphosphate (IP_3) and diacylglycerol (DAG), which regulate downstream calcium signaling. Surface expression of PI-PLC in *T. cruzi* occurs simultaneously with depletion of PIP_2 from host cells, host cytoskeletal changes, and calcium signaling (13). Therefore, the localization of PI-PLC in outer membrane lipid rafts of the flagellum may facilitate changes in the host during invasion.

Cytosolic calcium in *Trypanosoma* spp. regulates a number of important cellular processes such as host invasion by *T. cruzi* (38). Therefore, calcium-binding proteins are important for virulence. Flagellar calcium binding protein (FCaBP), a calcium binding protein in *T. cruzi*, is localized to lipid rafts, and its flagellar localization depends on binding of calcium ions (33). Calflagin Tb24, a calcium sensor in *T. brucei*, is also localized to lipid rafts (61). Mice infected with calflagin-deficient parasites survived for longer periods of time than mice infected with wild type parasites, indicating a role for calflagin in virulence (17). However, calflagin-deficient parasites were not altered in growth, morphology, motility, or ability to clear antibodies from their surface (17). Thus,

the role of calflagin in virulence is not precisely understood. The existence of distinct lipid raft domains within biological membranes has been previously proposed (57) and may also occur in protozoan parasites. The earliest study in parasites to support the existence of multiple raft domains was performed using membrane 'raft patching' in *Leishmania* spp. (14). This technique results in aggregated lipid raft domains. Both metacyclic lipophosphoglycan (LPG) and hydrophilic acylated surface protein B (HASPB) are DRM-associated in *Leishmania*, but do not co-localize to the same DRM 'patches' (14).

Similarly, other raft-isolation protocols have been used to support the existence of multiple lipid raft domains. Sucrose gradient fractionation is a widely accepted method for separating buoyant 'raft' from denser 'non-raft' fractions. Raft domains may span several fractions. In *P. falciparum*, the fractionation pattern for the detergent-resistant rhoptry protein, Pf34, differs from that of another detergent-resistant rhoptry protein, rhoptry-associated membrane antigen (RAMA), suggesting that multiple DRM populations exist within rhoptries (45). This is consistent with the observation that Pf34 is localized to the rhoptry neck while RAMA is localized to the rhoptry body (Figure 1) (45). Multiple lipid raft domains are also likely present in *E. histolytica*. The sucrose gradient flotation properties of the Gal/GalNAc lectin differs in parasites bound to RBCs as compared to those bound to collagen (24). In *L. major*, SMP-1, which is dually acylated, and another SMP protein, SMP-4, which is monoacylated, are also localized to distinct DRM fractions (58). While raft domains likely regulate cell signaling in these

parasites by segregating specific proteins, the existence of sub-populations of different types of rafts adds an additional level of control that may be important to virulence.

Host cell lipid rafts: more than just docks

Parasites manipulate host rafts to facilitate invasion. Although endocytosis is not a naturally occurring phenomenon in RBCs, *P. falciparum* induces the formation of the parasitophorous vacuole during invasion (40). As a means to study parasite modulation of host rafts, primaquine was used to induce endovesicular formation in RBCs. Primaquine-induced endovesicles are buoyant in sucrose gradients, cholesterol-rich, and contain proteins normally found in rafts, such as flotillin and stromatin, and may be isolated with non-detergent methods. Thus, they represent parasite-free, detergent-free, control endomembranes that may be compared to *P. falciparum* parasitophorous vacuoles. Primaquine-induced endovesicles contained a specific lipid profile, including phosphatidylserine and PIP_2; however, *P. falciparum*-induced vesicles did not harbor PIP_2. This evidence suggests that *P. falciparum* remodels RBC rafts during the invasion process (40).

Several studies have demonstrated the importance of host rafts during *T. cruzi* invasion. Chelation of cholesterol by MβCD or blocking of cholesterol synthesis in mammalian cells (macrophage, HEp2, HeLa, or Vero cells) interferes with adhesion and internalization of *T. cruzi* (4, 19, 44), implicating host lipid rafts in the attachment and invasion of *T. cruzi*. Host placental alkaline phosphatase (PLAP) can regulate internalization of *T. cruzi* (54). PLAP is a GPI-anchored protein that resides in DRM

microdomains (54, 54, 55, 55) and can be liberated by cholesterol chelation (44). Therefore, the loss of PLAP after cholesterol chelation may explain the inability of *T. cruzi* to invade MβCD-treated host cells (54). Exposure of HeLa or Vero cells to cholera toxin B subunit, which binds to ganglioside GM1, a marker of lipid rafts, or cholesterol chelating agents, MβCD or filipin, also inhibited invasion of both *T. cruzi* trypomastigotes and amastigotes, supporting the importance of rafts in host cell internalization of the parasite (4).

Similar to other host lipid rafts, host caveolae also serve as ports of entry for parasites. Caveolae are characterized by the presence of a family of proteins known as caveolins. Caveolin-1 knockout mice were employed to determine the role of caveolin-1 in the pathogenesis of *T. cruzi* (34). There was no difference in parasite load in the heart cells or macrophages of wild type or caveolin-1 null mice, suggesting that caveolin-1 is not essential for parasite entry or survival (34). By contrast, a separate study showed that caveolin-1 colocalizes with the point of contact between macrophages and trypomastigotes (4). In addition, during phagocytosis of metacyclic promastigotes by macrophages, parasites localize to areas of the macrophage membrane containing caveolin-1 (62). Thus, the role of caveolin-1 in parasite invasion is still being contended.

Despite the similarity in parasite-load, *T.* cruzi-infected caveolin-1 knockout mice did not survive as long as *T. cruzi*-infected wild type mice (34). Since caveolin-1 also regulates the release of chemokines, cytokines, and nitric oxide from immune cells, it is possible that an immune defect was responsible for increased virulence of the parasite in caveolin-1 knockout mice (34). Also, caveolin-3 levels were decreased in cardiac cells

after infection with *T. cruzi* (1). Caveolin-1/caveolin-3 double knockout mice exhibit symptoms of cardiomyopathy similar to that which is characteristic of Chagas‘ disease (43). This suggests that cardiac symptoms of Chagas‘ disease may be attributed to the effect of *T. cruzi* on host caveolae.

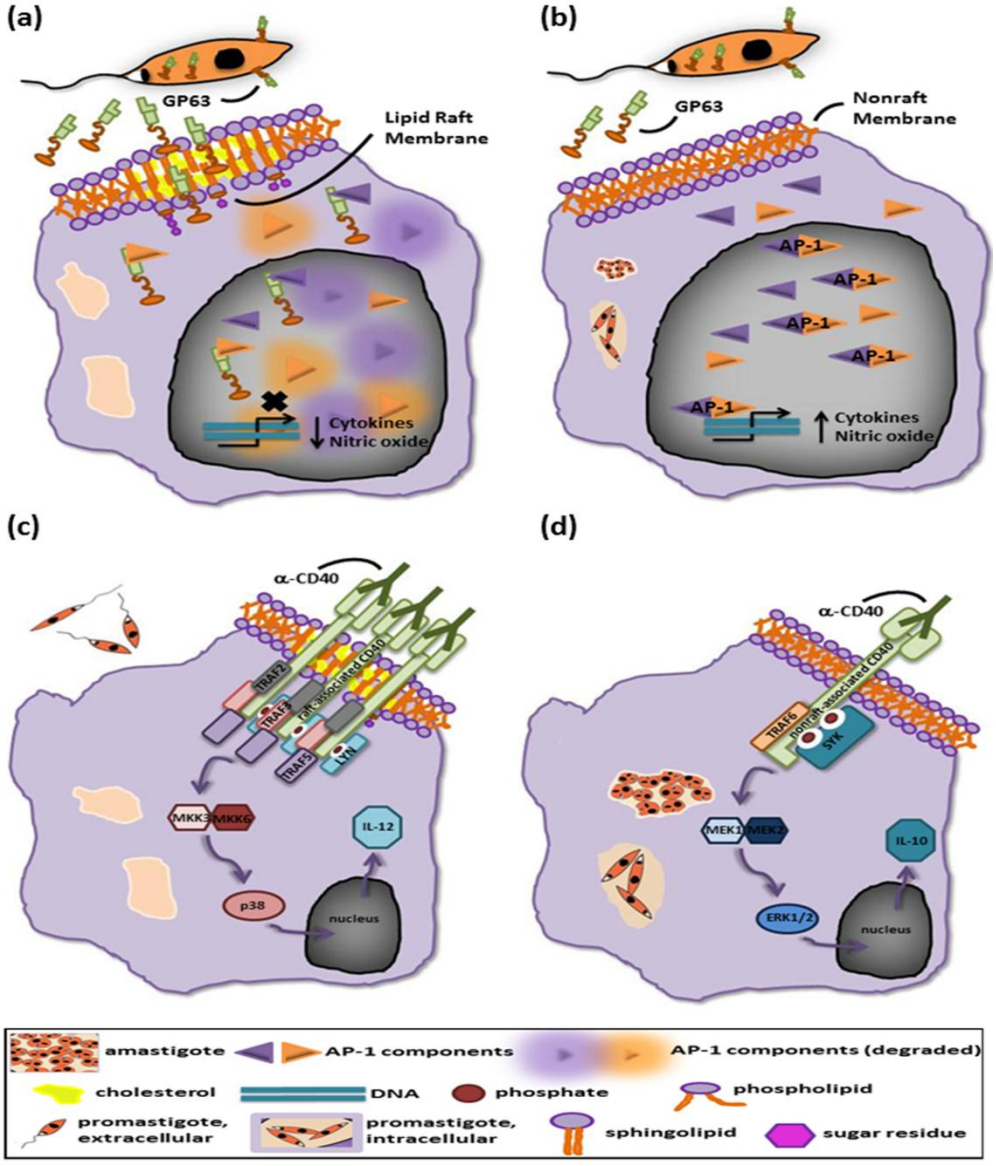
(a)
GP63
Lipid Raft Membrane
Cytokines
Nitric oxide
(b)
GP63
Nonraft Membrane
AP-1
AP-1
AP-1
AP-1
AP-1
Cytokines
Nitric oxide
(c)
α-CD40
TRAF2
TRAF3
TRAF5
LYN
MKK3
MKK6
IL-12
p38
nucleus
(d)
α-CD40
TRAF6
SYK
MEK1
MEK2
IL-10
ERK1/2
nucleus
amastigote
AP-1 components
AP-1 components (degraded)
cholesterol
DNA
phosphate
phospholipid
promastigote, extracellular
promastigote, intracellular
sphingolipid
sugar residue

Figure 2. *Leishmania* infections modulate the host immune response. (a) Early in infection, *Leishmania* promastigotes release GP63, a prominent surface metalloprotease. GP63 consists of an N-terminal catalytic domain, a central domain, and a C-terminal GPI-anchor, and it is localized inside parasites, at the parasite surface, and/or in a soluble, secreted form. GP63 enters macrophages via an undefined pathway that is phagocytosis-independent and lipid raft dependent, and can be found inside the cell cytoplasm and in the nucleus. The GPI-anchor may be involved in cellular entry via lipid raft membrane domains, since a GPI-less form of GP63 is minimally internalized in macrophages. GP63 actively degrades five of the seven subunits associated with the AP-1 transcription factor, essentially abolishing AP-1 activity (degradation is represented by halos surrounding the AP-1 subunits). Without AP-1, the expression of cytokines such as TNF-α, IL-1B, and IL-12, and the precursor of NO, iNOS, is severely reduced or abolished (8). **(b)** However, cholesterol chelation (and raft disruption) by MβCD or by intracellular *Leishmania* parasites results in partial inhibition of c-Jun degradation by GP63 (8). **(c)** In uninfected macrophages, stimulated raft-associated CD40 receptor triggers a signaling cascade that results in the production of the pro-inflammatory cytokine IL-12. Upon binding to CD40 ligand (or in this case, α-CD40), TRAF2, TRAF3, and TRAF5 are recruited to CD40, along with the Src family kinase, LYN, within lipid rafts. LYN activates MKK-3 and/or MKK-6, which, in turn, results in the phosphorylation and activation of p38. Changes in gene expression stimulated by p38 result in the production of IL-12, a cytokine that promotes infection suppression (22). **(d)** In macrophages infected with *L. major*, a different CD40- mediated signaling cascade is stimulated. Non raft-associated CD40 is complexed with TRAF6, along with the Syk family kinase, SYK. SYK activates MEK-1 and/or MEK-2, which phosphorylates and activates ERK1/2. The gene expression alterations attributed to ERK1/2 activation result in up-regulation of the anti-inflammatory cytokine, IL-10. *Leishmania* infections are promoted by IL-10 release. Interestingly, *L. major* chelates host cholesterol in a manner equivalent to treatment with MβCD, theoretically disrupting lipid raft domains. Since localization within rafts likely results in receptor clustering and conformational changes, it is possible that this explains the apparent differences in ‘signalosomes‘ utilized by uninfected and *Leishmania*-infected macrophages (22). In both **(b)** and **(d)**, the precise parasite stage that causes cholesterol chelation was not clearly identified, and therefore is represented by intracellular promastigotes and amastigotes.

Maintaining stowaway status: avoiding phagolysosomal acidification

Parasites may also manipulate host cell lipid rafts or caveolae in a manner that allows for evasion of the host cell lysosomal pathway (29), and interestingly, this may occur in a parasite stage-specific manner. For example, *L. i. chagasi* promastigotes require intact host caveolae for entry into macrophages and post-invasion replication (64). When promastigotes enter the host through caveolae, fusion of the vesicles containing promastigotes with lysosomes is delayed by 24-48 hours (48, 49). By contrast, the entry and survival of the amastigote form of *L. i. chagasi* is not affected by the loss of host caveolae, nor do amastigotes depend on this alternative route to avoid fusion with the lysosome, since they are better adapted to deal with phagolysosomal conditions (48). Likewise, in *T. cruzi*, phagocytosis of metacyclic, but not avirulent *T. cruzi* promastigotes, is associated with delayed parasitophorous vacuole-lysosomal fusion, and intracellular survival is enhanced (4). This suggests that particular life cycle stages of *Leishmania* and *T. cruzi* require intact lipid rafts or caveolae to evade lysosomal processing for survival.

Leishmania spp. also use other mechanisms to evade the host lysosomal pathway. *L. donovani* promastigotes transfer LPG from their membranes to the membrane of macrophages, where it disrupts lipid rafts and prevents F-actin assembly. This, in turn, disrupts phagosomal maturation and results in reduced phagocytosis rates of additional parasites (15, 66). Specifically, LPG insertion into macrophages inhibits the recruitment of the exocytosis regulator synaptin V to the nascent phagosome (63, 64). Synaptin V is required for the recruitment of vacuolar ATPase, which is responsible for

phagolysosomal acidification (15). Exclusion of synaptin V is beneficial for the parasite because cytotoxic acidification is prevented.

Safe harbor: evading the host immune system

Parasites must also evade the host immune system to survive. One way parasites accomplish this is through direct manipulation of host immune response. *Leishmania* spp. secrete the metalloprotease GP63, which is then taken up by host macrophages through their lipid raft domains (8, 25). Internalization of GP63 in host macrophages is associated with cleavage of the subunits of the early AP-1 signalosome such as C-Jun (8). This disrupts anti-microbial activity of macrophages (Figure 2) (8). In addition to the AP-1 transcription factor, GP63 cleaves host cell protein tyrosine phosphatases (PTPs), which regulate IFN-γ signaling in macrophages (25, 26). Cleavage of PTPs is lower in macrophages infected by GP63-null *L. major* (25). Additionally, disruption of macrophage lipid rafts by MβCD inhibits cleavage of PTPs by GP63 (25). Together, these data demonstrate the importance of GP63 in modulating immune cell activities.

L. donovani infection of macrophages causes disruption of membrane lipid rafts and changes in membrane fluidity (6). In antigen presenting cells such as macrophages, CD1d glycoproteins are responsible for signaling that leads to antigen presentation and the activation of T cells and natural killer cells (5). In uninfected macrophages, CD1d is present in lipid rafts (6). In *L. donovani*-infected macrophages, CD1d becomes non-raft associated (6); since *L. donovani*-infected cells do not express altered levels of CD1d, it is possible that the non-raft localization of CD1d was due to cholesterol chelation by

parasites. Likewise, in *L. major* infected macrophages, disruption of lipid rafts causes CD40 to localize to non-raft membrane (Figure 2) (50). When CD40 is raft-associated, it promotes the assembly of an IL-12-promoting CD40 signalosome, which suppresses *Leishmania* infection (50). Mislocalization of CD40 to non-raft membrane promotes the assembly of an IL-10-inducing CD40 signalosome which enhances *L. major* infection (50). IL-12 activates natural killer cells and induces T cell differentiation, which, in turn, promotes pro-inflammatory pathways leading to suppression of infection. IL-10 production, on the other hand, promotes an anti-inflammatory response, which supports *Leishmania* infection.

Targeting lipid rafts for disease management: all hands on deck

Lipid rafts have been identified as putative anti-parasite drug targets. Interestingly, targeting chemotherapeutic agents to rafts may increase their effectiveness (46). Additionally, key lipids in parasite rafts have subtle compositional differences as compared to mammalian lipids, making them excellent drug targets (68). Specifically in mammalian cells, sphingolipids are important for membrane structure and cell signaling (68). However, many parasites utilize unique inositol-based sphingolipids, including inositol phosphorylceramide (IPC); therefore, the enzymes, such as IPC synthase, which are required for biosynthesis of these unique lipids, could be targeted by novel chemotherapeutics.

Several studies have investigated the influence of existing drugs on parasite rafts. When *Giardia* was treated with β-lapachone, lipid raft staining was altered, suggesting

that raft domains were disrupted (9). Two drugs, sitamaquine and miltefosine, were tested for interaction with *Leishmania* rafts (7, 51). The presence of parasite rafts was essential for miltefosine activity. Wild type and miltefosine-resistant *Leishmania* parasites were stripped of sterols by incubation with cholesterol oxidase or MβCD (51). In both cases, drug susceptibility in wild type and mutant cells was reduced, and membrane sterol repletion restored drug sensitivity (51). A biomimetic membrane model was used to demonstrate that condensed domains (rafts) incorporated more miltefosine than fluid phase membrane domains, and this so called 'membrane reservoir' was likely essential for appropriate miltefosine internalization (51). However, another anti-*Leishmania* drug, sitamaquine, did not interact with sterols, and sterol depletion by cholesterol oxidase treatment did not significantly affect parasite drug susceptibility (7). It remains to be seen whether sitamaquine interacts with other *Leishmania* raft components, such as IPC.

The effects of chemotherapeutics on host cell rafts have also been investigated. For example, amphotericin B (AmB) sequesters cholesterol and prevents host cell binding by *Leishmania* (41). Although the precise mechanism is not yet understood, host cell receptors are important for *Leishmania* invasion. The researchers proposed that disrupted receptor signaling and function that was attributed to raft perturbation is responsible for their observations (41). Furthermore, cholesterol-rich domains are required endocytic entry points for some pathogens; AmB Amphotericin B mediated cholesterol sequestration may reduce *Leishmania* invasion by eliminating these domains (41).

In some cases, host raft disruption by drugs helped researchers understand host-parasite interactions. For example, lidocaine, a local anesthetic, reversibly disrupted erythrocyte lipid rafts without affecting membrane cholesterol content (30). Invasion of lidocaine-treated erythrocytes by *P. falciparum* parasites was inhibited in a dose-dependent manner (30). The specific mechanism of action of lidocaine allowed researchers to discern that disruption of raft specific signaling pathways, rather than membrane cholesterol content *per se*, was likely important in host cell invasion (30).

Concluding remarks

It is clear that both parasite and host lipid rafts participate in the virulence programs of eukaryotic pathogens. Parasites ensure their transmission and survival as stowaways by entering host cells through host rafts and/or by altering the architecture and function of host lipid microdomains. Disruption of parasite rafts inhibits adhesion, invasion, motility, and secretion. These parasite functions are all essential for infection. Although much has been learned about the importance of lipid rafts in parasite biology and virulence, there exist There are still a number of questions left to be answered concerning the role of lipid rafts in parasite biology and virulence (Box 2). Furthermore, there is evidence that chemotherapeutic raft disruption can alter parasite infectivity and/or drug susceptibility. However, in-depth understanding of drug interaction with parasite and/or host cell lipid rafts will be necessary for novel anti-parasitic drug design. Overall, our understanding of infection and immunity has undoubtedly been improved by new insights into lipid rafts—the ‘life rafts’ of parasites.

Box 1. Protein modifications and raft lipids

Lipid rafts contain a subset of proteins and lipids found in the plasma membrane. Certain post-translational modifications, such as GPI-anchoring and acylation facilitate protein-raft interaction. The lipids found in rafts, such as sphingolipids, are also involved in maintaining raft formation and stability.

GPI-anchored proteins are commonly localized to rafts, but it is unclear why particular GPI-anchored proteins exhibit this localization pattern. In *Leishmania* spp., both GP63 and LPG are GPI-anchored in all life cycle stages; however, GP63 is localized to the DRM in both procyclic and metacyclic promastigotes and LPG is localized only to DRM in metacyclic promastigotes (14). Although their subcellular localization differs, the GPI anchor of LPG in both parasite life-stages is identical (14); it is currently unknown what factors contribute to raft versus non raft localization of LPG.

In parasites, several studies have focused on the post-translational modifications of raft associated proteins and the lipids found in rafts. The localization of *T. cruzi* PI-PLC to flagellar membrane lipid rafts depends on dual acylation (13). The calcium sensors, FCaBP (*T. cruzi)* and calflagin Tb24 (*T. brucei),* depend on dual acylation for raft localization (33). Palmitoyl acetyl transferases (PATs) are responsible for the addition of palmitoyl groups to proteins; tbPAT7 palmitolylates *T. brucei* calflagin (18).

Flagellar proteins in *Leishmania major* are targeted to lipid rafts through post translational modifications. SMP-1, a small dually acylated protein, is targeted to flagellar rafts (59, 60). SMP-2 and SMP-4 are monoacylated (myristoylated) and localized to the flagellar pocket and cell body, respectively (58, 59). Like SMP-1, SMP-4 is

associated with DRM; however, SMP-2 is solubilized in detergent (59). Thus, dual acylation is not necessarily a raft targeting signal. Re-introduced SMP-1 to double knockout SMP-1 and SMP-2 cells was protective against sphingolipid depletion (59). This demonstrates that the role SMP-1 plays a role in stabilizing flagellar DRM (59).

Sphingolipids are a major component of lipid rafts. Therefore, researchers have investigated whether sphingolipids are necessary for raft formation in parasites. RNAi-mediated inhibition of serine palmitoyltransferase (SPT2) prevents *T. brucei* sphingolipid biosynthesis. Knockdown of SPT2 in procyclic-stage parasites did not affect calflagin localization to flagellar rafts; however, exposure of bloodstream *T. brucei* to myriocin, which also inhibits serine palmitoyltransferases, causes loss of association of calflagins with DRMs (20). Differences in membrane composition between the parasite stages, including the inclusion of ergosterol in procyclic forms, may account for these differences. Ergosterol may allow procyclic rafts to be more resistant to the removal of sphingolipids from their membranes (20). Lipid rafts in *Leishmania* spp. are also able to form in sphingolipid-deficient parasites, possibly due to the presence of ergosterol in *Leishmania* (14). Disruption of sphingolipid biosynthesis in *L. major* by myriocin has no effect on SMP-1 localization in rafts (60). Knockout of LmLCB2, a subunit of one serine palmitoyltransferase in *Leishmania*, yields parasites that cannot synthesize sphingolipids or ceramide. This deletion delays the association of GP63 with DRMs and changes the localization of LPG from non-raft to raft-associated (14). These changes affect the ability of the parasite to form infective metacyclic promastigotes (14).

Box 2. Outstanding questions: key unresolved questions about the role of lipid rafts in parasite biology and virulence

• **What is the exact composition of parasite lipid rafts and how does it differ from the composition of host lipid rafts?** Since parasite lipid rafts contain unique lipids (e.g., inositol phosphorylceramide (IPC) (68) and ergosterol (14)), which are not found in host lipid rafts, a more detailed analysis of the lipoid building blocks of parasite rafts is needed. Such information may be used to design new drugs that target the biosynthetic pathways of unique parasite lipids.

• Parasites depend on host lipid rafts for adhesion and invasion, but, this dependence seems to be parasite stage-specific (19, 25). **What regulates this stage-reliance on host lipid rafts and how can this be exploited for disease management?**

• **Do multiple sub-types of lipid rafts exist in parasite membranes?** There is evidence suggesting that diverse lipid rafts domains exist in individual parasites. This prediction is based on the non-overlapping localizations of DRM proteins (presumably lipid raft proteins) in whole cells (14) or sucrose gradients (24, 45, 58). Identification of distinct lipid rafts and discerning their unique functions will provide significant insight into protein trafficking in parasites.

• **To what extent does manipulation of host lipid rafts by parasites contribute to parasite survival?** The ability of parasites to remodel host lipid microdomains may represent an interesting strategy for enhancing parasite survival. Parasites may remodel host membrane through secreted factors (e.g., GP63 (8, 25)) or by surface bound factors (e.g., LPG (15, 63, 66)). Alterations to host lipid rafts by parasites may disrupt PIP_2-based signaling (40), interrupt the function of caveolae (1), inhibit antigen presentation (6), and promote specific cytokine signaling pathways that are beneficial to the parasite (50). However, it remains to be seen if other functions, such as host cell apoptosis or reactive oxygen synthesis, are also inhibited when host lipid rafts are remodeled by parasites.

• In addition to the general questions outlined above, there are also specific outstanding research questions for individual parasites. **For example, in *E. histolytica*, do lipid rafts regulate the assembly of the Gal/GalNAc lectin trimer? Do lipid rafts regulate cellular functions, other than glideosome-based motility in *T. gondii*? What specific adhesins are affected in *Giardia* after treatment with the raft disrupting agent, MβCD?**

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CHAPTER TWO

EXPOSURE TO HOST LIGANDS CORRELATES WITH CO-LOCALIZATION OF GAL/GALNAC LECTIN SUBUNITS IN LIPID RAFTS AND PHOSPHATIDYLINOSITOL (4,5)-BISPHOSPHATE SIGNALING IN *ENTAMOEBA HISTOLYTICA*

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Abstract

Entamoeba histolytica is an intestinal parasite that causes dysentery and liver abscess. Parasite cell surface receptors, such as the Gal/GalNAc lectin, facilitate attachment to host cells and extracellular matrix. The Gal/GalNAc lectin binds to galactose or N-acetylgalactosamine residues on host components, and is composed of heavy (Hgl), intermediate (Igl), and light (Lgl) subunits. Although Igl is constitutively localized to lipid rafts (cholesterol-rich membrane domains), Hgl and Lgl transiently associate with this compartment in a cholesterol-dependent fashion. In this study, trophozoites were exposed to biologically relevant ligands to determine if ligand-binding influences the submembrane distribution of the subunits. Exposure to human red blood cells (hRBCs) or collagen, *bonafide* Gal/GalNAc lectin ligands, was correlated with enrichment of Hgl and Lgl in rafts. This enrichment was abrogated in the presence of

galactose, suggesting that direct lectin-ligand interactions are necessary to influence subunit location. Using a cell line that is able to attach to, but not phagocytose, hRBCs, it was shown that physical attachment to ligands was not sufficient to induce the enrichment of lectin subunits in rafts. Additionally, the mutant had lower levels of PIP_2; PIP_2 loading restored the ability of this mutant to respond to ligands with enrichment of subunits in rafts. Finally, intracellular calcium levels increased upon attachment to collagen; this increase was essential for the enrichment of lectin subunits in rafts. Together, these data provide evidence that ligand-induced enrichment of lectin subunits in rafts may be the first step in a signaling pathway that involves both PIP_2 and calcium signaling.

Introduction

Entamoeba histolytica is an intestinal parasite responsible for dysentery and amebic liver abscess (22). Amebiasis is a food- and waterborne illness and is prevalent in underdeveloped countries lacking proper sanitation practices. As of 2010, it is estimated that 2.6 billion people worldwide do not use modern sanitation practices, and 886 million do not have access to clean drinking water sources (54). Thus, there is considerable global risk for acquiring *E. histolytica* infection.

Amebiasis occurs when food or water, contaminated with the environmentally resistant cyst form of the parasite, is ingested; excystation leads to the release of amoeboid trophozoites in the small intestine. Trophozoites then move to and colonize the large intestine. Serious complications arise when trophozoites invade the colonic

epithelium, enter the bloodstream, and travel to extra-intestinal sites such as the liver, lungs, and brain. During colonization of the host, trophozoites attach to numerous ligands, including red blood cells (RBCs), extracellular matrix (ECM) components (e.g., collagen and fibronectin), intestinal flora, colonic mucins, and leukocytes (6, 15, 39). Therefore, adhesion is an important virulence function for the parasite.

In mammalian cells, integrins are dimeric transmembrane receptors that are responsible for cell-cell and cell-ECM adhesion and signal transduction. Although no integrin homologs have been identified in the *E. histolytica* genome (27), attachment to ligands in the host can occur through cell surface receptors, which share sequence homology with integrins. One such receptor is the heterotrimeric protein complex, the galactose/N-acetylgalactosamine lectin (Gal/GalNAc lectin). This adhesin binds to galactose and N-acetylgalactosamine residues on host cells, and is composed of heavy (Hgl), light (Lgl), and intermediate (Igl) subunits. Hgl is a transmembrane protein that is disulfide linked to a glycophosphatidylinositol (GPI)-anchored Lgl. The heterodimer noncovalently associates with a GPI-anchored Igl. Both Hgl and Igl share sequence homology with β integrins (12, 46-48, 51), suggesting that they may also play a role in signaling.

Attachment of *E. histolytica* to human red blood cells (hRBCs) or collagen is inhibited in the presence of galactose, suggesting that the Gal/GalNAc lectin is an important receptor for these ligands (2, 33). On the other hand, binding of amoebae to fibronectin is not significantly inhibited by galactose, suggesting that the Gal/GalNAc

lectin may not be the major receptor for this ligand (33). The functional regulation of the Gal/GalNAc lectin is not well-understood.

In other systems, lipid rafts play a role in regulating the function of cell surface receptors, including integrins (24). Lipid rafts are tightly packed cholesterol- and sphingolipid-rich membrane microdomains. Lipid rafts are thought to serve as platforms within which signaling proteins interact. The removal of cholesterol, resulting in the disruption of lipid rafts, significantly inhibits the adhesion of *E. histolytica* trophozoites to host cells (23) and collagen (33), but only slightly inhibits the adhesion of trophozoites to fibronectin (33). This suggests that *E. histolytica* lipid rafts play a significant role in binding to host cells and collagen and a lesser role in binding to host fibronectin. The parallel roles of the Gal/GalNac lectin and lipid rafts in binding to collagen, but not fibronectin, suggest that these membrane domains regulate the function of the lectin.

In addition to protein receptors, lipids can also participate in signaling pathways that emanate from lipid rafts. One such family of signaling lipids are the phosphoinositides. Two phosphorylated members of the phosphoinositide family are phosphatidylinositol (4,5)-bisphosphate (PIP_2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Both of these lipids play important roles in cellular processes such as phagocytosis, protein kinase activation, and actin polymerization (9, 18). PIP_2 also regulates calcium signaling (8, 19, 28). For example, signal transduction can lead to hydrolysis of PIP_2, resulting in the production of second messenger molecules, inositol trisphosphate (IP3) and diacylglycerol (DAG) (20). These, in turn, facilitate the release of

calcium into the cytoplasm from intracellular calcium stores and from the extracellular space through channels in the plasma membrane (16, 40).

Phosphoinositides can also facilitate signaling by recruiting downstream proteins that have specific phosphoinositide binding domains. For example, FYVE-finger domains, which were originally observed in Fab1p, YOTB, Vac1p, and EEA1 proteins, bind specifically to phosphatidylinositol 3-phosphate (44). Additionally, certain pleckstrin homology (PH) domains, such as that from Bruton's tyrosine kinase (PH^{BTK}), have been shown to specifically bind PIP3 (42). Overexpression of GFP-FYVE-finger domains or GFP-PH^{BTK} domains have been used to localize phosphoinositides in real-time in *E. histolytica* (4, 38).

Previously, we demonstrated that cholesterol-loading of parasite membranes induced the enrichment of the Gal/GalNAc lectin subunits in lipid rafts, which, in turn, increased the activity of the Gal/GalNAc lectin (53). In this study, we have examined the localization of Gal/GalNAc lectin subunits after attachment to biologically relevant extracellular ligands. We show that binding to human red blood cells (hRBCs) and collagen results in the enrichment of Hgl and Lgl in lipid rafts, while attachment to fibronectin does not change the localization of the subunits. We also demonstrate that cells expressing GFP-PH^{BTK} exhibit reduced PIP_2 levels. In these cells, attachment to ligand is not correlated with enrichment of Hgl and Lgl in lipid rafts; the phenotype is reversible upon the addition of exogenous PIP_2, indicating a role for PIP_2 in regulating the submembrane position of the Gal/GalNAc lectin. Finally, intracellular calcium levels increase upon attachment to collagen; increased intracellular calcium levels appear to be

essential for the enrichment of lectin subunits in rafts. Together, our data suggest that co-localization of Gal/GalNAc lectin subunits in rafts may be the first step in the activation of a signaling pathway and that PIP_2 and calcium may be involved in this pathway.

Materials and Methods

Strains and culture conditions

E. histolytica trophozoites (strain HM1:IMSS) trophozoites were grown axenically in TYI-S-33 media (11) in 15 mL glass screw cap tubes or T25 cell culture flasks (Sarstedt, Newton, NC) at 37°C. The construction of a cell line conditionally expressing GFP-PH^{BTK} (tetracycline-inducible) is described elsewhere (4). GFP-PH^{BTK}-expressing trophozoites were maintained in TYI-S-33 media supplemented with 6 μg/mL G418 and 15 μg/mL hygromycin. The expression of GFP-PH^{BTK} was induced with 5 μg/mL tetracycline for 24 hr prior to use in assays. Prior to performing assays, cells were incubated on ice for 10 or 20 min in order to release them from tube or flask surfaces, respectively.

Exposure to Ligands

3.5×10^6 wildtype cells or GFP-PH^{BTK}-expressing cells were incubated in serum-free media for 30 min and then exposed to various ligands prior to lipid raft extraction. For hRBC exposure, trophozoites were incubated in the presence of 3.5×10^8 hRBCs (U.S. Biological, Swampscott, MA) for 5 min at 37°C. For exposure to collagen and

fibronectin, cells were incubated on ECM-coated flasks (BD Biosciences, Bedford, MA) or uncoated flasks (Sarstedt) for 15 min at 37^{o}C.

Lipid Raft Extraction

After exposure to ligands, isolation and characterization of lipid rafts were carried out as previously described (23). Extracted raft-associated proteins were characterized by SDS-PAGE and western blot as described previously (23). Primary antibodies included a mixture of monoclonal anti-Lgl antibodies (3C2, IC8, IA9, ID4) (1:4000 dilution), polyclonal anti-Hgl antibodies (1:5000 dilution), monoclonal anti-Hgl antibodies (1G7)(1:1000 dilution), or a mixture of monoclonal anti-Igl antibodies (3G5-A3-G3, 5H1-F11-D11, 4G2-D8-H1) (1:4000 dilution) (Antibodies were kind gifts from Dr. William Petri Jr., University of Virginia, 147 Charlottesville, VA). Western blots were analyzed by densitometry using ImageJ software 148 (Version 1.42q; U.S. National Institutes of Health, Bethesda, MD).

Whole Cell PIP_2 Extraction and Lipid Dot Blots

Total lipid was extracted from wildtype and GFP-PH^{BTK}-expressing trophozoites according to the methods of Gray *et al.*, (14). Briefly, 1 x 10^6 cells were washed twice with PBS. Lipids were precipitated by the addition of 5 mL of 0.5 M TCA and centrifuged at 500 x g for 5 min at 4^{o}C. The pellets were washed with 3 mL of 5% (w/v) TCA, 1 mM EDTA and centrifuged at 500 x g for 5 min. To the pellets, 3 mL of methanol:choloroform (2:1) was added and the mixture was vortexed 3 times over a

period of 10 min at room temperature to facilitate neutral lipid extraction. The extracted lipids were centrifuged at 500 x g for 5 min at 4°C. To the pellet, 2.25 mL methanol:chloroform:12.1N HCl (80:40:1) was added and the mixture was vortexed 4 times over 15 min at room temperature and centrifuged at 500 x g. The resulting supernatant was subjected to phase split by the addition of 750 μL chloroform and 1.35 mL 0.1N HCl. The solution was centrifuged at 500 x g for 5 min at 4°C. After centrifugation, the organic phase was collected and dried using a MiVac Duo Sample Concentrator Speed Vac centrifuge (GeneVac, Gardiner, NY).

The vaccum dried lipid pellets were resuspended in a methanol:chlorform:water mixture (2:1:0.8) and vortexed for 30 sec followed by sonication in a cold water bath for 10 min. The lipids were then spotted onto a nitrocellulose membrane. The membrane was blocked with 1.5% fatty acid-free BSA for 1 hr at room temperature and probed with mouse anti-PIP_2 (Abcam, Cambridge, MA) antibodies. Densitometric analysis was performed using Image J software.

PIP_2 Loading

GFP-PH^{BTK}-expressing cells were loaded with PIP_2 using a shuttle PIP_2 kit (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's guidelines. Concentrations of 25 μM PIP2 and 12.5 μM PIP_2 carrier histone (H1) were used. Loading was carried out for 30 min at 37°C. PIP_2 loading was confirmed using fluorescence microscopy of a BODIPY-labeled PIP_2 (Nikon Eclipse TI-E spectral confocal

microscope, Nikon Instruments Inc., Lewisville, TX). After PIP_2 loading, cells were exposed to hRBCs and lipid rafts were extracted as described above.

Calcium assay

Relative intracellular calcium levels were assessed using the calcium indicator, fluo-4/AM, according to the manufacturer's instructions. Fluo-4/AM is fluorescent when bound to calcium. Wildtype cells were washed twice with calcium stain loading buffer (CSB) (50), and then incubated in CSB supplemented with 5 μM fluo-4/AM (Invitrogen, Carlsbad, CA) or an equivalent volume of DMSO (diluent control) for 30 min at 37C. After staining, cells were washed twice with CSB, and 1 x 10^5 cells (stained or control) were added to the wells of a 12-well plate, which contained 1 mM $CaCl_2$ (5) and a glass coverslip, coated with collagen or fibronectin (BD Biosciences). After 3 min, plates were transferred to a BioTek Flx800-I microplate reader (BioTek, Winooski, VT), incubated at 37C, and fluorescence (excitation 485 nM, emission 525 nM) was monitored at 5 min intervals for 10 min. To account for background or fluorescence, the fluorescence value of control cells (DMSO) was subtracted from the fluorescence value of fluo-4/AM-stained cells.

Calcium chelation

To chelate intracellular calcium, cells were incubated in the presence of 50 μM 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) BAPTA/AM (EMD Chemicals Group, Darmstadt, Germany) in serum free media for 30

min at 37^{o}C. Cells were then exposed to collagen coated coverslips and the calcium assay was performed as described above. Cells were also exposed to collagen coated flasks and lipid rafts were isolated and characterized as described above.

Adhesion assay

To determine the effect of intracellular calcium chelation on adhesion, we used a previously described adhesion assay (33, 38). Cells were pre-exposed to serum free media with or without 50 μM BAPTA/AM for 30 min at 37^{o}C in the presence of the fluorescent vital stain calcein-AM (5 μg/mL). 3 x 10^4 cells were seeded in the wells of a 96-well collagen coated plate (BD Biosciences) (in triplicate) for each condition and incubated at 37^{o}C for 15 min. The wells were then washed with warm PBS to remove non-adherent cells. Fluorescence was measured using a BioTek Flx800-I microplate reader (excitation 485 nM, emission 525 nM). Values were reported as percent of control, which is arbitrarily set to 100%.

Statistical Analysis

All data are reported as a mean ± S.D.. Statistical analyses were carried out using GraphPad Instat V.3. Comparisons were carried out using a one way ANOVA with post-test. *P* values less than 0.05 (*) were considered significant, and values less than 0.01 (**) or 0.001 (***) were considered highly statistically significant.

Results

Exposure to hRBCs correlates with enrichment of Hgl and Lgl subunits in lipid raft fractions

In mammalian cells, binding to ligand induces clustering of integrins in lipid raft domains (17). To determine if ligand engagement also influences the submembrane distribution of the subunits of the Gal/GalNAc lectin, we exposed trophozoites to hRBCs, and isolated and characterized lipid rafts as described (23). The composition of lipid rafts confers detergent-resistance to these membrane domains. Therefore, purification of lipid rafts was initiated by extraction with cold triton X-100. This resulted in the isolation of detergent-resistant membrane (DRM), which consists of both lipid raft and actin-rich membrane. Since the buoyant density of lipid rafts is less than that of actin-rich membrane, these two membrane domains were further separated by sucrose density gradient centrifugation. To address possible contamination of DRM from hRBCs, whole cell lysate from hRBCs were tested by western blot with antibodies for Hgl, Lgl, and Igl and were shown to have no cross reacting proteins (Appendix Figure D-1).

Western blot analysis of gradient fractions revealed that the majority of Igl was found in a low density region (fractions 9-14) (Figure 1). Previously, these fractions were shown to possess the highest levels of cholesterol as compared to other detergent resistant fractions (23). Thus, these fractions are identified as lipid rafts. The localization of Igl to these low density rafts was consistent with previous reports (23, 53). In control cells, the majority of Hgl and Lgl was associated with less buoyant, actin-rich fractions (fractions 17-20) (Figure 1). However, after exposure to hRBCs, there was an increase in the

proportion of Hgl and Lgl that was localized to lipid raft fractions (fractions 9-14), whereas the sub-membrane distribution of Igl remained unchanged (Figure 1). This observation suggests that binding to at least one ligand, hRBCs, can induce the enrichment of Hgl and Lgl in lipid rafts.

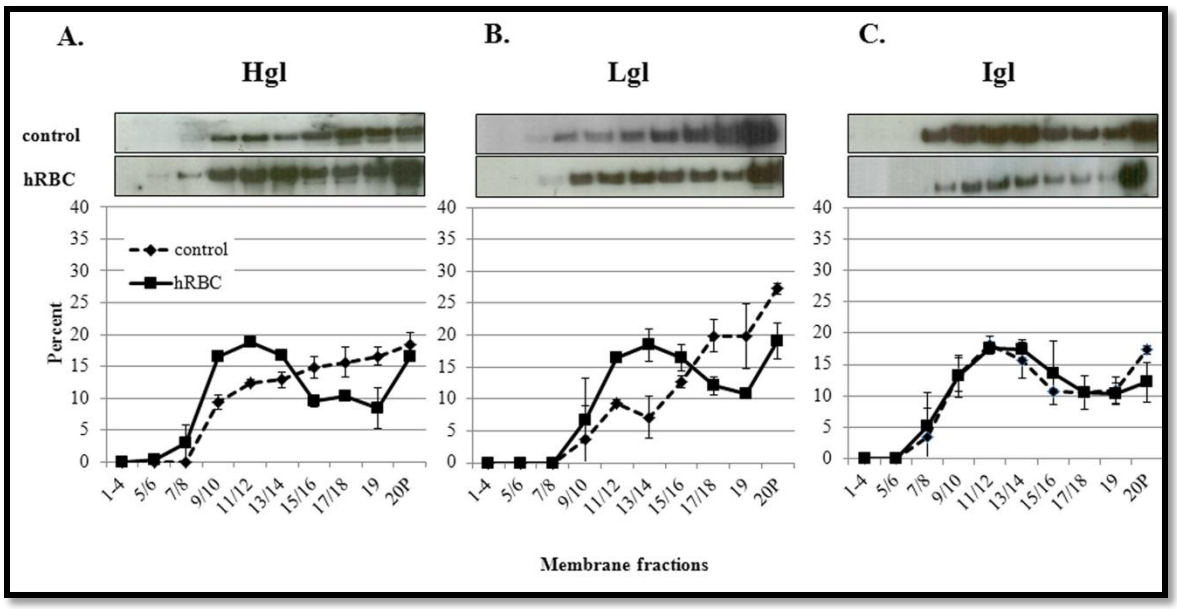

Figure 1. Exposure to hRBCs is correlated with enrichment of Hgl and Lgl in lipid rafts. Trophozoites (3.5 x 10^6) were serum-starved and exposed to hRBCs. Detergent-resistant membrane (DRM) was extracted and fractionated using sucrose gradient density centrifugation (SGDC). Nineteen fractions and a pellet (20P) were collected and subjected to SDS-PAGE and western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). In both control trophozoites and trophozoites exposed to hRBCs, Igl is predominantly localized to fractions 9-14, previously identified as lipid rafts. Hgl and Lgl, which are localized to dense, actin-rich fractions 17-20 in control cells, are enriched in lipid rafts (fractions 9-14) upon hRBC exposure.

To determine if enrichment of Hgl and Lgl in lipid rafts was dependent on a physical interaction between the Gal/GalNAc lectin and its ligand, cells were pre-treated with galactose, a competitive inhibitor of lectin-ligand binding, or mannose (a control sugar), prior to hRBC exposure. Incubation with galactose prevented the enrichment of Hgl and Lgl in lipid raft fractions after hRBC exposure, while incubation with mannose did not inhibit the enrichment of Hgl and Lgl in lipid raft fractions after hRBC exposure (Figure 2). The localization of Igl in lipid raft domains was unaffected in the presence of galactose or mannose. These data suggest that physical interaction between the Gal/GalNAc lectin and its ligand is necessary for raft enrichment of Hgl and Lgl.

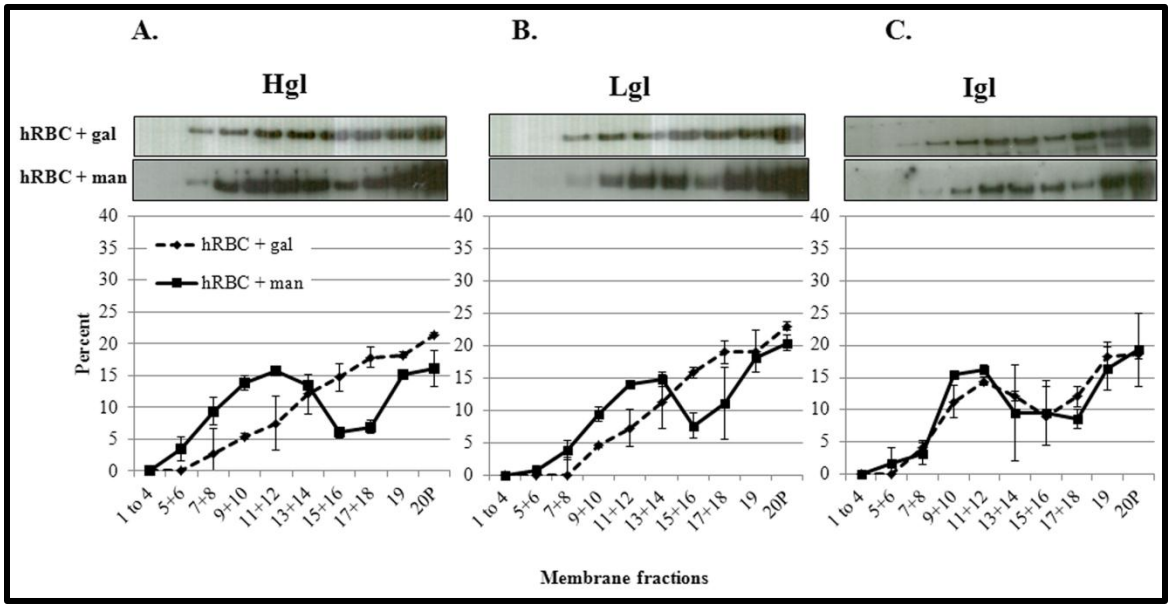

Figure 2. Enrichment of Hgl and Lgl in lipid rafts upon exposure to hRBCs is inhibited in the presence of galactose. Trophozoites (3.5×10^6) were serum-starved and exposed to 10 mM galactose (gal) or 10 mM mannose (man), prior to exposure to hRBCs. DRM was isolated and fractionated using SGDC. Nineteen fractions and a pellet (20P) were collected and subjected to western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). The localization of Igl remained unchanged after exposure to galactose or mannose in followed by hRBCs. The enrichment of Hgl and Lgl in lipid rafts after exposure to hRBCs was inhibited in the presence of galactose, but not mannose.

Exposure to collagen type I correlates with galactose-sensitive enrichment of Hgl and Lgl subunits in lipid rafts

To determine if another ligand also induces the enrichment of Gal/GalNAc lectin subunits in lipid rafts, we exposed trophozoites to collagen type I, which has been shown to initiate signaling in *E. histolytica* (7, 10, 35). Trophozoites were incubated on collagen-coated flasks or uncoated control flasks. Lipid rafts were extracted and characterized. Similar to incubation with hRBCs, incubation on collagen was accompanied by an increase in the levels of Hgl and Lgl subunits in high buoyancy lipid raft fractions (Figure 3). Interestingly, the fractions with the highest levels of Hgl and Lgl (fractions 13-16) (Figure 3) differed from those with the highest levels of Hgl and Lgl after exposure to hRBCs (fractions 9-14) (Figure 2). This suggests that the molecular mechanism governing the submembrane distribution of the Gal/GalNAc lectin subunits differs in a ligand-specific manner. This enrichment was prevented by the addition of galactose, but not by the addition of mannose (Figure 4). Therefore, physical interaction of trophozoites with collagen also appears to be necessary for enrichment of Hgl and Lgl in lipid rafts.

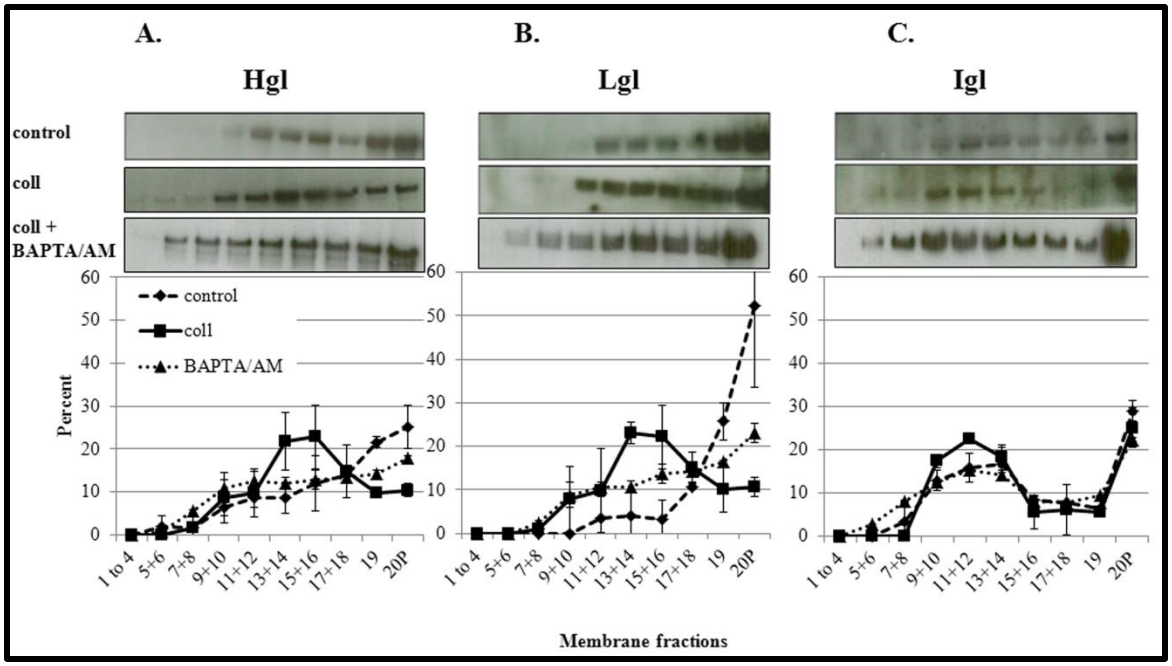

Figure 3. Exposure to collagen is correlated with a calcium dependent enrichment of Hgl and Lgl in lipid rafts. Trophozoites (3.5×10^6) were serum-starved or incubated in the presence of BAPTA/AM, and incubated on collagen-coated flasks or uncoated control flasks. DRM was isolated and fractionated by SGDC. Nineteen fractions and a pellet (20P) were collected and subjected to western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). In cells treated with collagen, the distribution of Igl was not different from that in control cells. Hgl and Lgl subunits were enriched in fractions 13-16 upon exposure to collagen. Enrichment of Hgl and Lgl in lipid rafts was inhibited in the presence of BAPTA/AM.

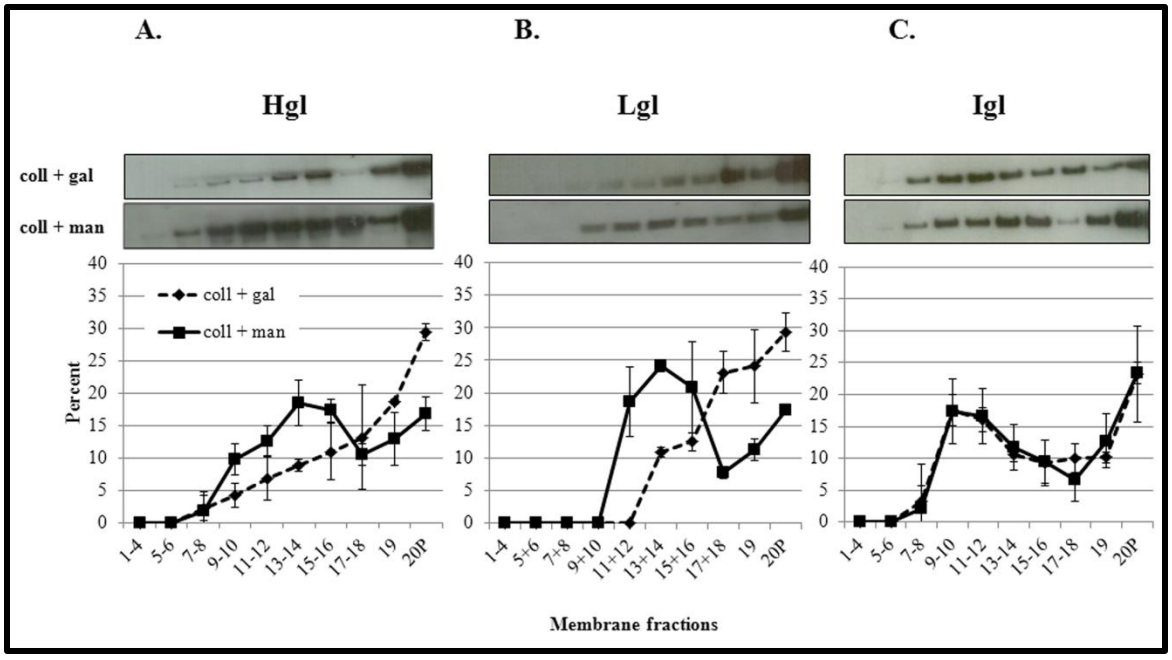

Figure 4. Enrichment of Hgl and Lgl in lipid rafts after exposure to collagen is inhibited by presence of galactose, but not by mannose. Trophozoites (3.5×10^6) were serum-starved and pre-treated with 10 mM galactose (gal) or 10 mM mannose (man). Cells were then incubated on collagen-coated flasks for 15 min at 37oC. DRM was isolated and subjected to SDGC. Nineteen fractions and a pellet (20P) were collected and subjected to western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). The localization of Igl to lipid rafts (fractions 9-14) remained unchanged in the presence of galactose or mannose. The enrichment of Hgl and Lgl in lipid rafts after exposure to collagen was inhibited in the presence of galactose but not mannose.

Exposure to fibronectin does not correlate with an enrichment of Hgl and Lgl subunits in lipid rafts

Because galactose and raft-disrupting agents have little effect on trophozoite-fibronectin interaction (33), it is likely that neither the Gal/GalNAc lectin nor lipid rafts play a primary role in the interaction between the parasite and this ECM component. Therefore, as a control, we incubated trophozoites on fibronectin-coated flasks, and isolated and characterized lipid rafts. In both control cells and cells exposed to fibronectin, Hgl and Lgl were concentrated in the actin-rich fractions (fractions 17-20), while Igl was concentrated in lipid raft fractions (fractions 9-14) (Figure 5). Therefore, exposure to fibronectin did not affect the localization of any of the Gal/GalNAc lectin subunits, and in particular, did not induce the enrichment of Hgl and Lgl in lipid raft domains of *E. histolytica*. This supports the authenticity of our finding that binding to a *bonafide* ligand of the Gal/GalNAc lectin (e.g., hRBCs and collagen) can influence the submembrane localization of this adhesin.

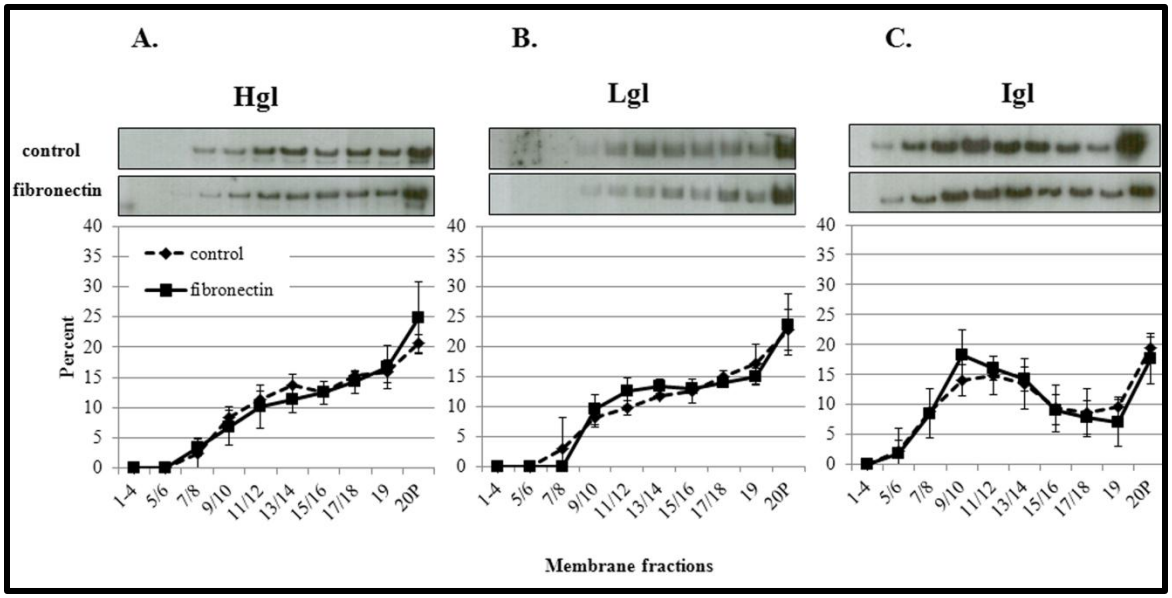

Figure 5. Exposure to fibronectin is not associated with enrichment of Gal/GalNAc lectin subunits. Trophozoites (3.5 x 10^6) were serum-starved and incubated on fibronectin-coated flasks, or uncoated control flasks. DRM was isolated and fractionated using SDGC. Nineteen fractions and a pellet (20P) were collected and subjected to western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). In both control and fibronectin exposed cells, Igl was localized to fractions 9-14, previously identified as lipid rafts. Hgl and Lgl were primarily localized to fractions 17-20 in both fibronectin-exposed and control cells.

Attachment to hRBCs is not sufficient for enrichment of Hgl and Lgl in lipid rafts

Previously, an *E. histolytica* cell line expressing (GFP)-labeled PH domain derived from Brutons Tyrosine Kinase (GFP-PH^{BTK}) was developed (4). The GFP-PH^{BTK}-expressing cell line exhibited interesting phenotypes, including enhanced motility and a phagocytic defect characterized by the ability to bind to, but not internalize, hRBCs (4). The latter characteristic provided the opportunity to test the sufficiency of ligand binding in the regulation of Gal/GalNAc localization. GFP-PH^{BTK}-expressing cells were exposed to hRBCs, and lipid rafts were purified and characterized. In this cell line, attachment to hRBCs was not correlated with the enrichment of Hgl and Lgl in lipid rafts (Figure 6), suggesting that while necessary (Figures 2, 4), ligand binding is not sufficient to induce enrichment of Hgl and Lgl in lipid rafts.

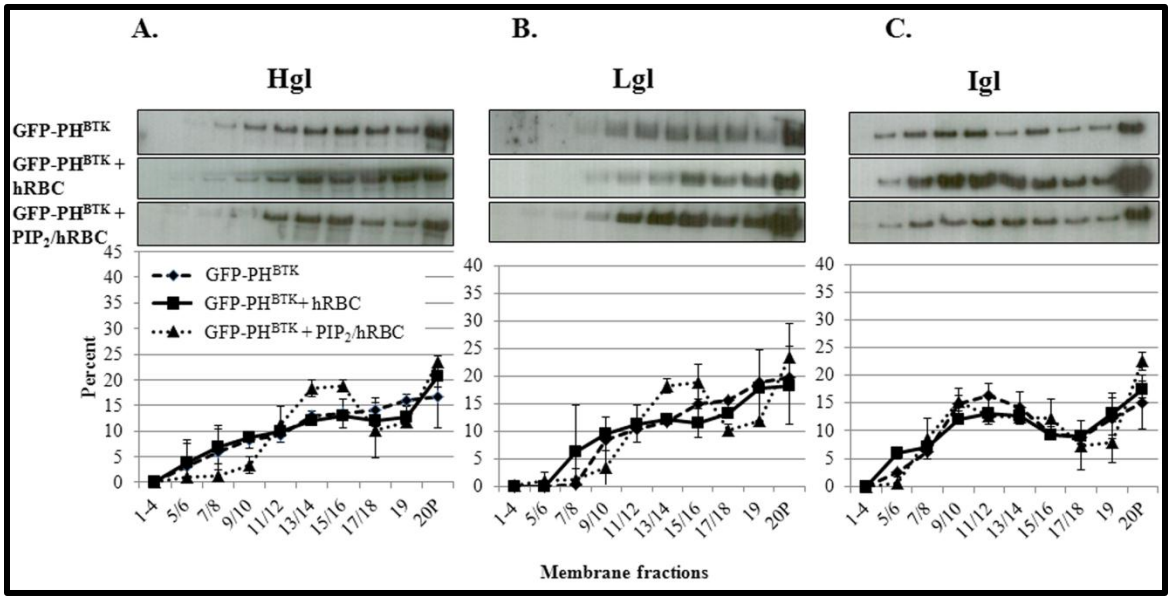

Figure 6. PIP_2 plays a role in Hgl and Lgl enrichment in lipid rafts. GFP-PH^{BTK}-expressing trophozoites (3.5×10^6) were serum-starved and exposed to hRBCs. DRM was isolated and fractionated using SGDC. Nineteen fractions and a pellet (20P) were collected and subjected to western blot analysis using antibodies specific for (A) Hgl, (B) Lgl, or (C) Igl. Average values and standard deviations for densitometric scans (n=2) are reported as percent of total detergent-resistant protein for each subunit (± S.D.). In the mutant, the submembrane distribution of the three subunits remained unchanged upon exposure to hRBCs. PIP2 loading restored the enrichment of Hgl and Lgl in lipid raft fractions.

PIP_2 regulates the submembrane distribution of Hgl and Lgl

Given the phenotype of the GFP-PH^{BTK}-expressing cells (4), we hypothesized that phosphoinositide signaling was altered in the mutant. Therefore, we used lipid dot blots to determine the levels of PIP_2 in the transgenic cell line. Compared to wildtype cells, the level of PIP_2 in GFP-PH^{BTK}-expressing cells was decreased approximately 77% (Figure 7). Since the regulation of integrin function depends on PIP_2 signaling (21, 25, 26), it is conceivable that alterations in the levels of this lipids could influence the enrichment of Hgl and Lgl in lipid rafts upon ligand binding. In other systems, it has been established that PIP_2 resides in rafts (reviewed in reference (29, 37, 52). Since the GFP-PH^{BTK}-expressing cell line had reduced levels of PIP_2, we determined if addition of exogenous PIP_2 to this mutant could rescue the Hgl- and Lgl-raft enrichment defect. The mutant cell line was loaded with PIP_2 using a Shuttle PIP_2 kit (Echelon Biosciences), and the successful addition of PIP2 to cells was confirmed by fluorescence microscopy using BODIPY-labeled PIP_2 (Figure 7). Interestingly, loading of PIP_2 resulted in restoration of the ability of this cell line to respond to hRBC exposure with enrichment of Hgl and Lgl in lipid raft domains (Figure 6). However, PIP_2 addition did not completely reverse the phenotype since the percent enrichment of Hgl and Lgl in rafts was less in the PIP_2-loaded mutant than in rafts in wildtype cells (Figure 1). These data provide genetic evidence of a role for PIP_2 in regulating the lectin subunit localization in lipid rafts.

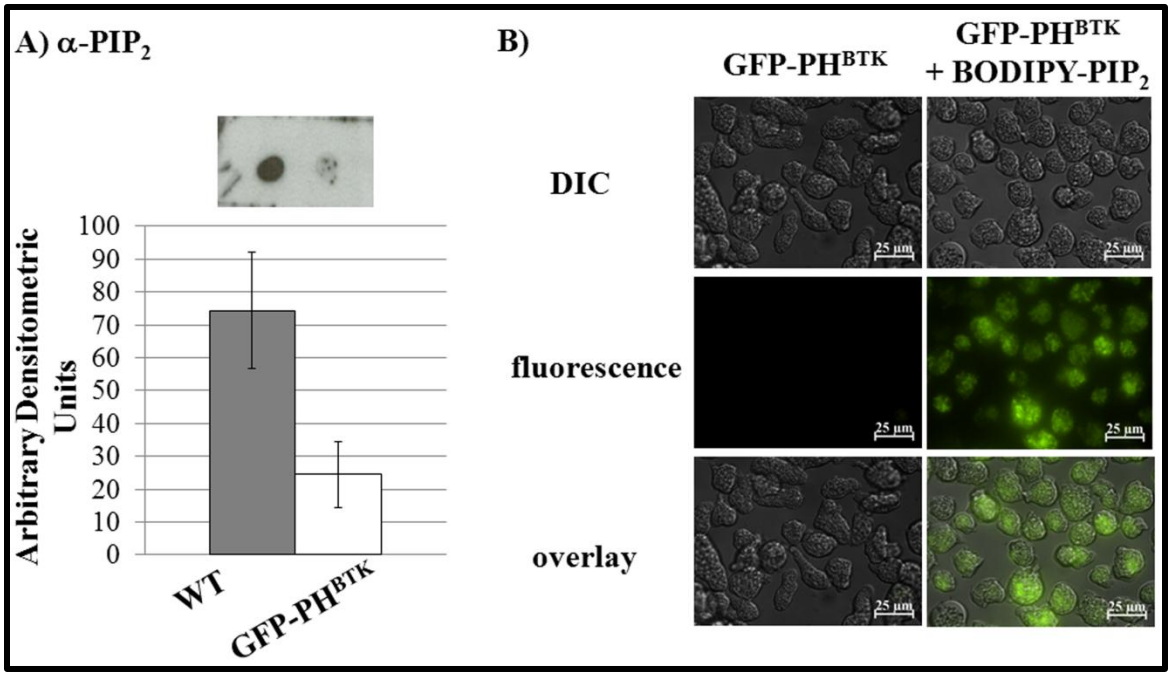

Figure 7. GFP-PH^{BTK}-expressing cells have altered PIP_2 levels, and can be loaded with PIP_2. A) Phosphoinositides were extracted from whole cell lysates and PIP_2 levels were measured using dot blots with antibodies specific PIP_2. Levels were analyzed and assigned a value of arbitrary densitometric units. PIP_2 levels were lower in GFP-PH^{BTK}-expressing cells as compared to wildtype cells. B) PIP_2 loading in GFP-PH^{BTK}-expressing cells was confirmed using a BODIPY-labeled PIP_2.

Calcium signaling is necessary for the enrichment of Hgl and Lgl in rafts after ligand binding.

In other systems, PIP_2 can be hydrolyzed into IP_3 and DAG, which facilitates calcium signaling (8, 43). Given the importance of PIP_2 in the localization of Hgl and Lgl to lipid rafts, we measured intracellular calcium levels after exposure to collagen and fibronectin using a fluorescence-based calcium assay. We observed a significant increase in intracellular calcium levels after exposure to collagen, but not after exposure to fibronectin (Figure 8).

To determine if the accumulation of intracellular calcium was essential for the localization of Hgl and Lgl in lipid rafts, we exposed trophozoites to BAPTA-AM, an intracellular calcium chelator, prior to exposure to collagen. Reduction of calcium by BAPTA-AM was confirmed using the fluorescence based calcium assay (Figure 8). Exposure to BAPTA/AM, prior to exposure to collagen, prevented the enrichment of Hgl and Lgl in lipid rafts (Figure 3), suggesting that the accumulation of intracellular calcium is necessary for lipid raft association of Gal/GalNAc lectin subunits. It is possible that the failure of Hgl and Lgl to become enriched in lipid rafts after exposure to BAPTA/AM and collagen was due to decreased adhesion. We measured adhesion to collagen in the presence of BAPTA/AM. Adhesion to collagen was not significantly inhibited in the presence of 50 μM BAPTA/AM (Figure 9). This suggests that any effects of BAPTA/AM exposure on intracellular calcium levels and the localization of Hgl and Lgl were not simply due to a decrease in adhesion to the collagen-coated surfaces.

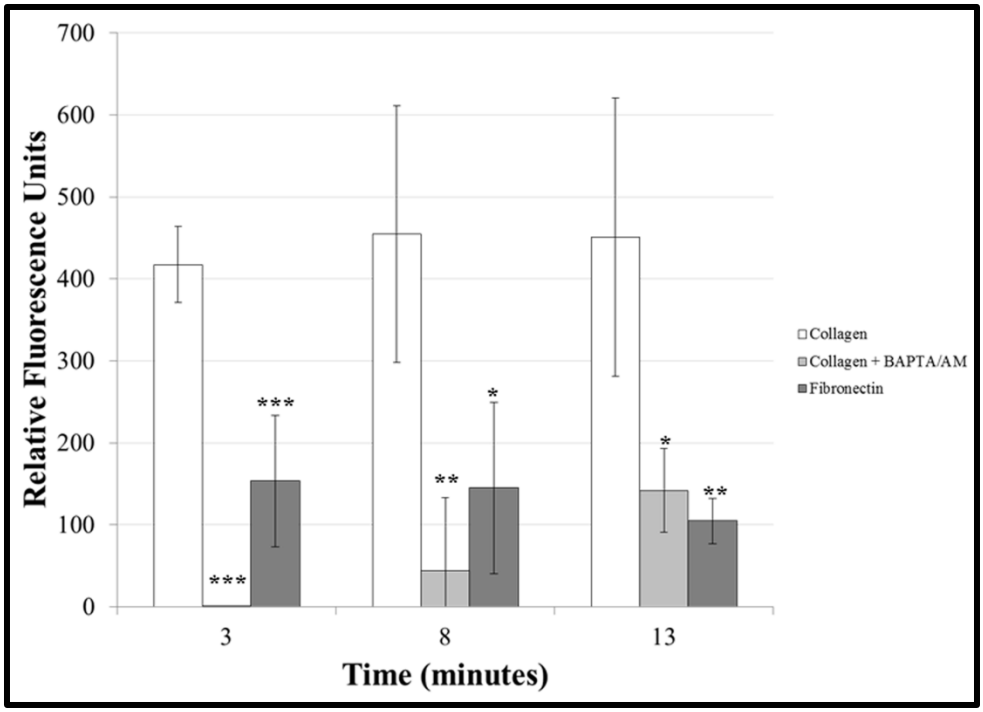

Figure 8. Intracellular calcium levels are significantly higher in collagen-exposed cells than in collagen/BAPTA/AM- or fibronectin-exposed cells. Intracellular calcium levels were measured for wildtype trophozoites that were exposed to collagen, with or without BAPTA/AM, or fibronectin. As compared to collagen-exposed cells (n=3), calcium levels in BAPTA/AM-exposed (n=4) or in fibronectin-exposed cells were significantly lower at all tested time points.

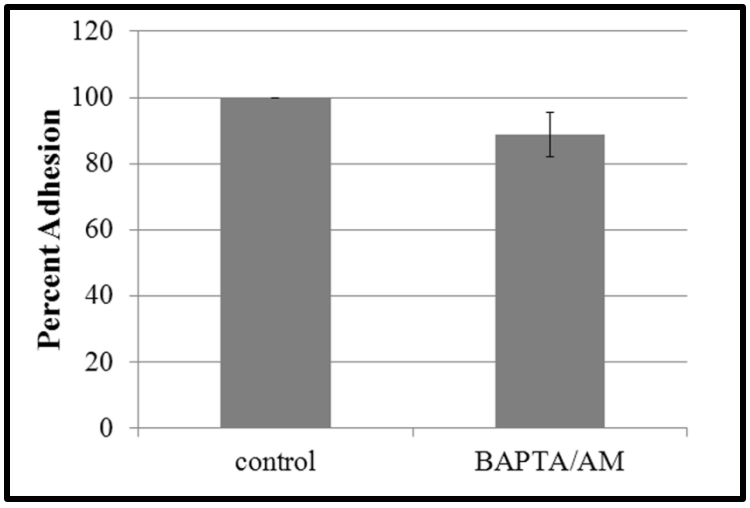

Figure 9. Adhesion to collagen is not significantly inhibited in the presence of BAPTA/AM. Adhesion to collagen was measured for wildtype cells that were exposed to serum free media with or without BAPTA/AM. Values were averaged and adhesion is represented as percent of control, set to 100% ± S.D. (n=3). Adhesion to collagen was not significantly inhibited in the presence of BAPTA/AM or control.

Discussion

In this study, we have shown that exposure of *E. histolytica* to *bonafide* Gal/GalNAc lectin ligands (e.g,. hRBCs or collagen) was accompanied by enrichment of the Gal/GalNAc lectin subunits, specifically Hgl and Lgl, in lipid raft domains. Previously, it was shown that cholesterol-loading induced co-localization of Gal/GalNAc lectin subunits in rafts and increased activity of the Gal/GalNAc lectin (53). Here, we have provided evidence that another condition, namely ligand binding, can also influence the sub-membrane localization of the Gal/GalNAc lectin subunits. We have also shown that binding to ligand was necessary, but not sufficient, to induce enrichment of Hgl and Lgl in lipid rafts after ligand binding. Our data also indicate that PIP_2 and calcium participate in the enrichment of Gal/GalNAc lectin subunits in rafts.

Enrichment of Hgl and Lgl in high buoyancy lipid raft domains after ligand binding is similar to the clustering and activation of mammalian integrins in lipid rafts. For example, in Jurkat T lymphocytes, attachment to collagen type IV or fibronectin induces lipid raft enrichment of α2β1 and α4β1 integrins, respectively (17). Furthermore, activation of another integrin in Jurkat T lymphocytes, lymphocyte function-associated antigen 1 (LFA-1), is correlated with its enrichment in lipid rafts (24). Although these signaling pathways are well understood in immune cells, the current study is an important first step towards the understanding of downstream signaling pathways that arise from lipid rafts in a parasite model.

The present study shows that attachment to ligand results in co-localization of the three lectin subunits in lipid raft fractions. Previously, it was shown by

immunoprecipitation that Igl associates with Hgl (30). Importantly, we have not shown that Hgl and Lgl physcially interact with Igl in lipid rafts. However, it is conceivable that the enrichment of Hgl and Lgl in raft regions, that already contain Igl, facilitates the assembly of the lectin into a functional trimer. This, in turn, may serve to activate subsequent raft-based signaling pathways related to virulence.

Exposure to hRBCs or collagen was correlated with the enrichment of Hgl and Lgl subunits in lipid rafts. Interestingly, these raft populations differed slightly in their buoyant density. For example, after binding to hRBCs, Hgl and Lgl associated with rafts that were more buoyant than the rafts harboring these same subunits after collagen-binding. It is possible that there are multiple types of lipid rafts within the parasite membrane, and binding to collagen or hRBCs causes the lectin to localize to distinct and separate lipid raft domains. In other systems, there is evidence for distinct raft populations. For example, purification of rafts from Madin Darby Canine Kidney cells, using a variety of detergents, resulted in the isolation of distinct lipid raft domains with different protein residents (41). Immunogold labeling and electron microscopy has shown that all lipid raft markers do not co-localize. These data from other systems support the notion that multiple lipid raft domains exist within the plasma membrane (55). Our data suggest that the same is true in *E. histolytica*.

Differences in the buoyant density of rafts containing the lectin may be due to the association of the lectin with a different set of signaling proteins or cytoskeletal proteins in a ligand-specific manner. In neutrophils, heavier detergent resistant membranes were found to contain more cytoskeletal proteins (34). Adhesion plaques, which contain actin,

myosin I and II, α-actinin, vinculin, and tropomyosin (49), have been observed in *E. histolytica* upon attachment to ECM components, but has not been observed upon attachment to hRBCs. Thus, the formation of a Gal/GalNAc lectin containing adhesion plaque after exposure to collagen may explain why the lipid rafts harboring the lectin after collagen exposure are less buoyant than those harboring the lectin after hRBC exposure.

We showed that ligand binding was not correlated with the enrichment of Hgl and Lgl in rafts in a transgenic cell line with reduced levels of PIP_2. We also showed that addition of exogenous PIP_2 to this cell line partially rescued the phenotype. Together, these data provide strong genetic evidence for a role for PIP_2 in regulating the submembrane distribution of the lectin subunits in *E. histolytica*. To our knowledge, this is the first study, in any system, to use a PIP_2 deficient mutant to illustrate the role of PIP_2 in protein-lipid raft interactions.

In the current study, intracellular calcium levels were increased upon exposure to collagen but not fibronectin. Others have shown that calcium levels increase when trophozoites are exposed to fibronectin (5). One explanation for this difference is that we exposed cells to fibronectin coated coverslips instead of fibronectin in solution (5); adhesion to the solid ECM surface may initiate different signaling pathways. It is currently unknown if the increased intracellular calcium levels are directly related to PIP_2 hydrolysis in the cell or are attributed to other mechanisms related to calcium influx. In mammalian cells, the physical interaction between αIIβ3 integrin, sodium-proton exchangers, and sodium-calcium exchangers occurs simultaneously with integrin binding

to ligand, and results in increased intracellular calcium levels (56). Additionally, in phagocytes, extracellular calcium influx was shown shown to be essential for movement of an integrin bound to adenylate cyclase toxin from *Bordetellae* into lipid rafts (3). Similarly, in the current study, the increase in calcium levels was shown to be necessary for ligand induced enrichment of Hgl and Lgl in lipid raft domains.

Other studies, in mammalian cells as well as in *E. histolytica*, have supported the connection between calcium, PIP_2, regulation of actin cytoskeleton, regulation of transcription, and virulence. For example, *E. histolytica* calcium signaling has been shown to activate transcriptional regulators, such as NF-κB and NFAT (13). Likewise, attachment to collagen by trophozoites induces an increase in the binding of transcriptional regulators, AP-1, STAT1, and STAT3 to DNA (7, 36) and an increase in the expression of several important virulence factors, including amoebapore and cysteine proteases (10). In *E. histolytica*, actin remodeling occurs during attachment to collagen (32) and hRBCs (1), and calcium mobilization can affect actin organization (5). In mammalian cells, calpain, a calcium-dependent protease has been shown to cleave the cytoskeletal elements talin, filamin, and α-actinin, thereby releasing integrins from the actin cytoskeleton (45). It has been proposed previously that this cleavage of talin may be responsible for freeing proteins to allow their recruitment to lipid raft domains (3). PIP_2 also contributes to actin cytoskeletal re-organization by guiding and activating actin binding proteins (20, 31). PIP_2 plays an important role in mammalian cells by binding to talin, thereby targeting it to focal adhesions where it can interact with and activate integrins (25). Together with our data, these findings suggest an intriguing link between

parasite-host interactions, raft association of the Gal/GalNAc lectin, calcium mobilization, the cytoskeleton, and changes in gene expression.

The data presented here provide insights into signaling pathways in *E. histolytica* and, importantly, add to a developing model of the regulation of Gal/GalNAc lectin function. In the absence of ligand, GPI-anchored Igl subunits predominantly reside in raft-like domains, whereas Hgl-Lgl dimers are primarily localized to a different sub-membrane compartment. Binding to at least two biologically relevant ligands, hRBCs and collagen, brings all three subunits to the same raft fractions. Interestingly, our data are the first to show a correlation between the sub-membrane position of the lectin subunits and phosphoinositide-based signaling in this pathogen. In the future, it will be important to identify effectors that act downstream and in parallel with the Gal/GalNAc lectin after ligand binding and enrichment in lipid rafts. Fully understanding the behavior of this receptor after contact with extracellular ligands during invasion is necessary to fully appreciate virulence functions in *E. histolytica.*

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CHAPTER THREE

THE ROLE OF POSTTRANSLATIONAL MODIFICATIONS AND PROTEIN-PROTEIN INTERACTIONS IN THE LOCALIZATION OF THE GAL/GALNAC LECTIN OF *ENTAMOEBA HISTOLYTICA*

Abstract

Entamoeba histolytica is a protozoan parasite which causes amebiasis and liver abscess in areas of the world with poor sanitation. Adhesion is an essential part of *E. histolytica* virulence. Cell surface receptors, such as the Galactose N-acetylgalactosamine lectin (Gal/GalNAc lectin) are responsible for attachment to host surfaces. The Gal/GalNAc lectin is composed of three subunits, heavy (Hgl), light (Lgl), and intermediate (Igl). Igl is constitutively localized to lipid rafts, which are cholesterol-rich and detergent-resistant microdomains. Hgl and Lgl become enriched in lipid rafts upon binding of host cell ligands; however, the cellular mechanisms by which raft-associated proteins, including Hgl and Lgl, become associated with these domains in *E. histolytica* are currently unknown. Therefore, we sought to explore both posttranslational modifications and protein-protein interactions that lead to the association of Hgl and Lgl with lipid rafts. Inhibition of N-linked glycosylation was shown to decrease levels of both Hgl and Lgl-35 (35 kDa isoform) in raft-fractions. Hgl was shown to be palmitoylated in whole cells and chemical inhibition of palmitoylation in *E. histolytica* was shown to reduce Hgl-raft

association. This indicates a possible role for palmitoylation in the localization of Hgl in lipid rafts. Genetic or chemical inhibition of GPI-synthesis was associated with enrichment of Hgl in rafts, indicating that interaction of Hgl with a GPI-anchored protein, either Lgl or other, regulates its submembrane localization. Overexpression of the cytoplasmic domain of Hgl also induced enrichment of endogenous Hgl in rafts indicating a role for the cytoplasmic domain of Hgl in raft-association. Therefore, we conclude that glycosylation and palmitoylation are positive regulators of the association of lectin subunits with rafts, while GPI-anchoring and protein-interaction with the cytoplasmic tail of Hgl are negative regulators of this association.

Introduction

Lipid rafts are tightly packed, cholesterol- and sphingolipid-rich regions within the cell membrane. Lipid rafts serve as signaling platforms within which protein-protein interactions are facilitated. In protozoan parasites, lipid rafts control adhesion, vesicle trafficking, motility, and cell signaling (reviewed in (16)). These raft functions are regulated, for the most part, by the proteins that reside in these domains.

There are a variety of mechanisms regulating the localization of proteins to rafts. Posttranslational modifications, protein-protein interactions, and specific domains within proteins regulate raft localization. In a study using giant plasma membrane vesicles, Levental *et al*. determined that the most common posttranslational modification on raft-enriched proteins was palmitoylation (12.4% of proteins) (23). Palmitoylation (also referred to as S-acylation or S-palmitoylation) is the addition of a 16-carbon, palmitic

acid to a cysteine residue. It is different from other fatty acid additions as it is (1) reversible, and (2) can be added at the plasma membrane level (reviewed in (36)). In both *Trypanosoma cruzi* and *Trypanosoma brucei*, dual acylation, which is the addition of palmitoyl and myristoyl groups, is necessary for targeting of calcium binding proteins to lipid rafts of the flagella (29).

Levental *et al*. also determined that the second most common posttranslational modification regulating protein raft-association is a GPI-anchor (11.2% of proteins) (23). GPI-anchors allow the incorporation of proteins into liquid ordered raft-domains (37). In fact, the replacement of the transmembrane domain of β-Secretase, with a GPI-anchor, changed its localization from non-raft membrane to lipid rafts (11). Additionally, glycosylation has been identified as a protein modification that can induce raft-association (6, 8, 47).

In addition to posttranslational modifications, interaction with other proteins, such as those of the cytoskeleton, has also been shown to regulate raft-association of proteins. The actin cytoskeleton is necessary for the formation of liquid order domains (reviewed in (10)). F-actin acts as a negative regulator of T cell integrin-raft association, including a lymphocyte associated antigen 1 (LFA-1) and α4β1 (22). On the other hand, depolymerization of actin in myoblasts prevents the association of N-cadherin, another adhesion molecule, with detergent-resistant rafts (7).

Entamoeba histolytica is a protozoan parasite for which lipid raft function is correlated with virulence (17, 20, 45). It is the causative agent of amebiasis and amebic liver abscess. Amebiasis occurs when food or water, contaminated with the

environmentally resistant cyst form of the parasite, is ingested. Trophozoites excyst in the small intestine and move to and colonize the large intestine. During colonization of the host, trophozoites adhere to host cells and host extracellular matrix; therefore, adhesion is an important virulence function for the parasite (21). In support of this, disruption of myosin-II based adhesion renders *E. histolytica* avirulent for liver abscess development (40). Furthermore, adhesion is necessary for other virulence functions, including phagocytosis and invasion (reviewed in (38)).

E. histolytica attaches to host cells and host extracellular matrix (ECM) through a variety cell surface receptors. The best characterized of these receptors is the galactose N-acetylgalactosamine lectin (Gal/GalNAc lectin) (34). The Gal/GalNAc lectin is so named because it binds to galactose or N-acetylgalactosamine residues on host cells and host ECM components. The Gal/GalNAc lectin is composed of three subunits: heavy (Hgl), light (Lgl), and intermediate (Igl). Hgl and Lgl are disulfide linked, while Igl is non-covalently associated with the heterodimer. Multiple isoforms of the subunits are expressed in *E. histolytica* cells. A recent annotation of the genome suggests that there are 5 isoforms of Hgl and 6 isoforms of Lgl (26). Two of the Lgl isoforms have been characterized. One of these isoforms is a 31 kDa (Lgl-31) GPI-anchored protein while the other isoform is a 35 kDa (Lgl-35), non-GPI anchored, heavily glycosylated protein (28). There may be as many as 30 isoforms of Igl; thus, genetic manipulations that target Igl may be more difficult than those that target the Hgl and Lgl subunits (39). In steady state, Igl is localized to lipid rafts while the Hgl-Lgl heterodimer is localized to non-raft, actin-rich membrane (17, 20, 45). Upon cholesterol loading of the membrane or

exposure to *bonafide* Gal/GalNAc lectin ligands, the Hgl-Lgl heterodimer becomes enriched in raft domains (17, 45); however, the mechanism by which these subunits become enriched in rafts is not known.

Since all three subunits are glycosylated (9, 28) this modification may be important in Gal/GalNAc lectin-raft interactions. The region between amino acids 482 and 818 of Hgl possesses potential glycosylation sites (28) and 24 hour exposure to tunicamycin, a nucleoside of bacterial origin that can inhibit N-glycosylation, reduces the the size of Hgl by approximately 10 kDa. Both forms of Lgl are predicted to be glycosylated; however, Lgl-35 is predicted to be heavily glycosylated (28). Hgl labels with palmitic acid (33) suggesting that it may also be palmitoylated. Igl (9) and Lgl-31 (28) are both GPI-anchored. Knockdown of GPI-anchoring in *E. histolytica* through antisense inhibition of EhPIG-M1 (phosphatidylinositol glycan mannosyltransferase) or EhPL-AS (GlcNac-phosphatidylinositol deacetylase) affected pathogenicity, adhesion, fluid-phase endocytosis, and ability to resist lysis by human serum (41, 44). Therefore, GPI-anchoring is important to *E. histolytica* virulence.

In *E. histolytica,* the cytoplasmic domain of Hgl has been shown to have motifs similar to those in the cytoplasmic tails of β2 and β7 integrins that regulate integrin signaling pathways (28, 42). Expression of a soluble cytoplasmic domain of Hgl decreased adherence to and cytolysis of CHO cells by trophozoites (28, 40, 42). Overexpression of HGL-2, a construct which contains only the transmembrane and cytoplasmic domain of Hgl, leads to decreased attachment to enterocytes (30). Thus, the

cytoplasmic tail of Hgl may regulate lectin activity through inside-out signaling. Whether the cytoplasmic tail regulates Hgl-raft interaction is currently not known.

In this study, we have performed a preliminary analysis of the mechanisms by which the lectin subunits become raft-associated. We have focused on the Hgl-Lgl dimer as its association with rafts is transient (17, 45). We have uncovered potential roles for glycosylation, palmitoylation, GPI-anchoring, actin, and the cytoplasmic tail of Hgl. This is the first study in *E. histolytica* to address the mechanisms by which to raft-localization occurs.

Methods

Cell culture and mutant cell lines

Entamoeba histolytica trophozoites (strain HM1:IMSS) were cultured axenically in TYI-S-33 media (12) in 15 mL glass screw cap tubes or 50 mL culture flasks at 37^{o}C. Wildtype trophozoites were electroporated with the Hgl2$_{SP\text{-}TM\text{-}COO^-}$ construct, which is an ectodomain truncation of Hgl in the pExEhNeo *E. histolytica* expression plasmid or an empty vector control (40). The resulting cell lines were named HGL-2 and NEO, respectively, and were maintained in 10 μg/mL G418. The plasmid encoding the antisense construce of Eh-PIG-M1 was also transfected into wildtype trophozoites as described above. These cells were maintained in 5 μg/mL hygromycin, as previously described, and exposed to tetracycline (1 μg/mL) for 5 days prior to experimentation as previously described (44). All plasmids were kind gifts of Dr. Nancy Guillen (Institut Pasteur, Paris, France).

Lipid raft extraction

Lipid rafts were extracted from wildtype or mutant cells as previously described (20). Briefly, 3.5 x 10^6 cells were exposed to ice-cold 0.1% (v/v) triton X-100 followed by sucrose density gradient centrifugation. Fractions were collected and proteins were precipitated using TCA. In some cases, raft fractions or actin-rich fractions were pooled. Extracted raft-associated proteins were characterized by SDS-PAGE and western blot as described previously (20). Primary antibodies included a mixture of monoclonal anti-Lgl antibodies (3C2, IC8, IA9, ID4) (1:4000 dilution), polyclonal anti-Hgl antibodies (Rabbit N-terminal antibody to amino acid sequence 88-110, Thermo Scientific, 1:5000 dilution), or a mixture of monoclonal anti-Igl antibodies (3G5-A3-G3, 5H1-F11-D11, 4G2-D8-H1) (1:4000 dilution) (Antibodies for Igl and Lgl were kind gifts from Dr. William Petri Jr., University of Virginia, 147 Charlottesville, VA). Western blots were analyzed by densitometry using ImageJ software 148 (Version 1.42q; U.S. National Institutes of Health, Bethesda, MD).

Inhibition of Posttranslational Modifications

Glycosylation was inhibited by exposing wildtype trophozoites to 5 µg/mL tunicamycin or DMSO (diluent control) for 24 hours as previously described (27). After a 24 hour exposure, raft-fractions and actin-rich fractions were purified and characterized. Palmitoylation was inhibited by the exposing wildtype trophozoites to 2-bromopalmitate (100 µM) (Sigma-Aldrich, St. Louis, MO) or 100% ethanol (diluent control) as previously described (13) for 24 hours prior to raft-extraction and characterization. GPI-

anchor inhibition was carried out by the exposing wildtype trophozoites to 0.5 mM phenylmethanesulfonylfluoride (PMSF) or isopropanol (diluent control) for 1 hour prior to raft-extraction and characterization.

Acyl Biotin Exchange

1 x 10^7 cells were lysed using 0.2% (v/v) triton x-100 for 30 min. Cell lysates were subjected to 2 freeze-thaw cycles in liquid nitrogen. Protein was extracted using TCA precipitation, and acyl biotin exchange was carried out on extracted protein as previously described (43). Briefly, the protein pellet was incubated in the presence of lysis buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 7.4) with 1 mM NEM, 1 × protease inhibitors (PI), 1 mM PMSF, and 0.2% triton X-100 overnight at 4^{o}C. NEM was removed from samples by three sequential chloroform-methanol extractions. Pelleted protein was re-suspended in 4SB (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) and divided into two equal proportions. One tube was used for +hydroxylamine reaction and the other as a negative control (-hydroxylamine). To the +hydroxylamine reaction, protein was incubated in +HA buffer (0.7 M hydroxylamine, 1 mM HPDP–biotin, 0.2% triton X-100, 1 mM PMSF, 1 × PI pH 7.4). For the –hydroxylamine reaction, protein was incubated in –HA buffer (50 mM Tris, 1 mM HPDP–biotin, 0.2% triton X-100, 1 mM PMSF, 1 × PI, pH 7.4). Samples were rotated for 1 hour at room temperature. Following treatment, samples were exposed to three chloroform methanol extractions, and resulting pellet was re-dissolved in 4SB. Samples were then incubated in the presence of low HPDP-biotin buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 0.2 mM HPDP–biotin,

0.2% triton X-100, 1 mM PMSF, 1 × PI, pH 7.4). Unbound proteins were removed by four sequential washes (lysis buffer, 0.1% SDS, 0.2% triton X-100), and bound proteins were released from resin by exposing the affinity resin to lysis buffer containing 0.1% SDS, 0.2% Triton X-100, and 1% β-mercaptoethanol. Samples were incubated for 15 min at 37 °C with occasional gentle mixing. Finally, samples were TCA precipitated by adding TCA to a 10% (v/v) final concentration. Samples were incubated on ice for 20 min, samples were centrifuged at 15,000g, 10 min, 4°C. Final pellet was dissolved in 30 µl 2SB (2% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4). Then dilute to 150 µl with lysis buffer. SDS-PAGE and western blots were performed as previously indicated.

Immunoprecipitation

For immunoprecipitation assays, 4 x 10^4 cells expressing HGL-2 were lysed using 0.2% (v/v) triton x-100 for 30 min. Cell lysates were frozen and thawed twice in liquid nitrogen. Cell lysates were then incubated by rotation with sheep anti-rabbit or sheep anti-mouse dynabeads (Invitrogen, Carlsbad, CA) for 2 hours at room temperature to pre-clear the mixtures. Following pre-clearing, lysate was incubated with either (concentration) α-FLAG (rabbit) or (concentration) α-Hgl (mouse) antibodies. To this mixture, sheep anti-rabbit or sheep anti-mouse dynabeads were added, and were rotated overnight at 4°C. Western blots were performed as described above.

Actin Depolymerization

To disrupt the actin cytoskeleton, 3 x 10^5 trophozoites were pre-treated with an actin disrupting agent, cytochalasin D (CytoD, Sigma–Aldrich, St. Louis, MO, USA), or an equivalent volume of CytoD diluent (DMSO), for 60 min at 37^{o}C prior to isolation and characterization of detergent-resistant membrane (DRM).

Fluorescence microscopy

Actin staining using the fluorescent actin probe, Alexa Fluor 488-phalloidin (Invitrogen), was carried out as previously described (46). Stained cells were viewed by confocal laser scanning microscopy using an LSM510 microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA).

Results

Glycosylation is important for the localization of Hgl and Lgl-35 in raft-domains

Since Hgl and Lgl are glycosylated (28, 33), we wanted to determine if glycosylation was necessary for their association with lipid rafts. Therefore, we employed tunicamycin and a previously published protocol that was shown to efficiently reduce this posttranslational modification in *E. histolytica* (28). During extraction of detergent resistant membrane (DRM), both raft-fractions (9-12) and actin-rich fractions (17-20P) were collected. Additionally, triton soluble supernatant (TSS) was collected and analyzed.

In tunicamycin exposed cells, the association of Hgl with rafts decreased while its association with actin-rich fractions increased (Figure 1). The level of Hgl remained unchanged in TSS (Figure 1). Exposure to tunicamycin also reduced the size of Hgl (Figure 1) as previously described (28). After exposure to tunicamycin, only the GPI-anchored, Lgl-31 subunit was localized with rafts. Non-GPI anchored Lgl-35, which was predicted to be highly glycosylated, disappeared from actin-rich membrane, although the total amount of Lgl in actin-rich membrane did not decrease (Figure 1). The higher molecular weight bands of Lgl, normally seen on western blots, were present in the TSS of both treated and untreated cells (Figure 1); but, there was no difference in the total level of Lgl in either tunicamycin-treated or control protein levels in TSS. Although we cannot rule out the possibility that inhibition of glycosylation on proteins other than Hgl and Lgl were responsible for changes in the submembrane location of Hgl and Lgl, these data suggest that glycosylation directly or indirectly regulates the association of Hgl and Lgl-35 with rafts.

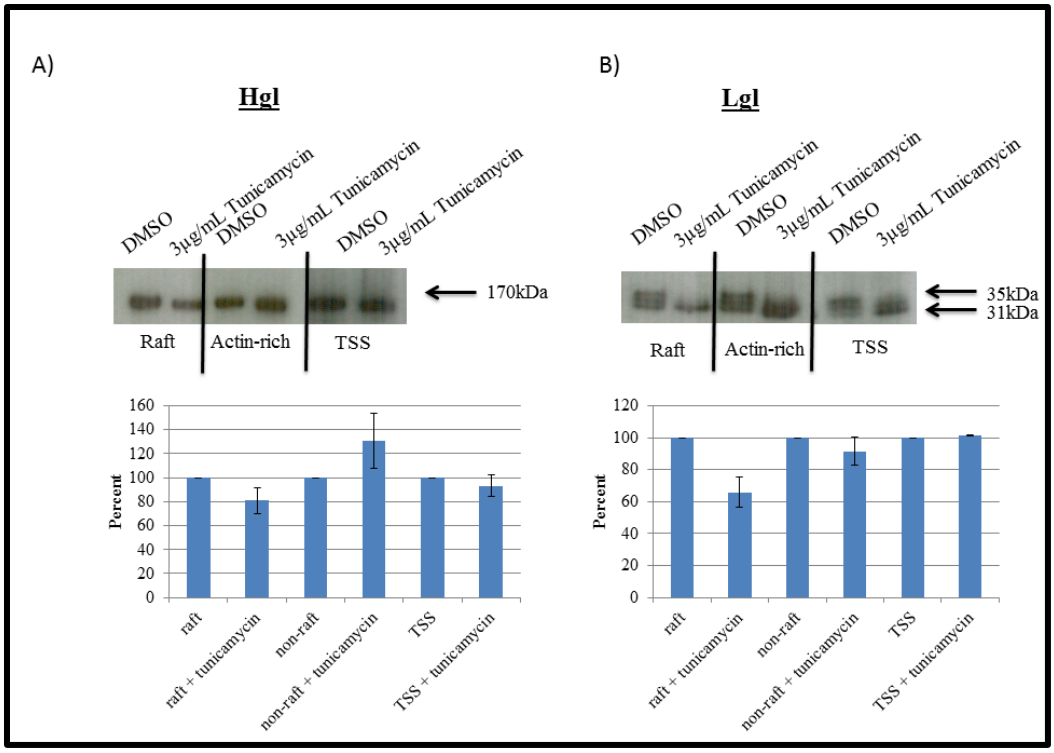

Figure 1. Inhibition of protein glycosylation inhibits Hgl and Lgl protein localization in raft-fractions. Trophozoites were exposed to 3 µg/mL tunicamycin or an equivalent volume of DMSO (control) for 24 hours prior to raft extraction. Fractions were pooled as raft (9-12) or actin-rich (17-20P) and triton soluble supernatant (TSS) and subjected to western blotting and densitometry. The data represent mean densitometric units (±S.D.) of 2 trials and are reported as percent of DMSO control for (A) Hgl and (B) Lgl which was arbitrarily set to 100%

Hgl is palmitoylated, and palmitoylation appears to regulate Hgl-raft association

Since palmitoylation is the only major raft-targeting posttranslational modification that can be added to proteins at the plasma membrane (24), palmitoylation may be responsible for the movement of Hgl (and consequently Lgl) from non-raft to raft domains. Since Hgl labels with palmitic acid, we wanted to confirm that Hgl was, in fact, palmitoyled using acyl-biotin exchange (ABE) (33). ABE has been used to characterize palmitoylation and the palmitoylation proteome in yeast, mammalian cells, and a protozoan parasite, *Trypanosoma brucei* (13, 14, 43). Therefore, it represents a widely used method for examining this posttranslation modification. We extracted protein from *E. histolytica* and used ABE to determine the palmitoyl status of Hgl in untreated whole cell lysates. Hgl was shown to be palmitoylated (Figure 2). This confirmed previous findings (33) and validated the use of this protocol in *E. histolytica*.

We next wanted to assess the role of palmitoylation in the localization of Hgl to DRM using 2-bromopalmitate, which blocks the addition of palmitate to proteins. Exposure of *E. histolytica* to 2-bromopalmitate resulted in a decrease in the level of Hgl in raft fractions (Figure 3). Although we cannot rule out the possibility that inhibition of palmitoylation on proteins other than Hgl and Lgl were responsible for changes in the submembrane location of Hgl; these data provide evidence that palmitoylation is involved, in some way, in the localization of Hgl in lipid raft domains.

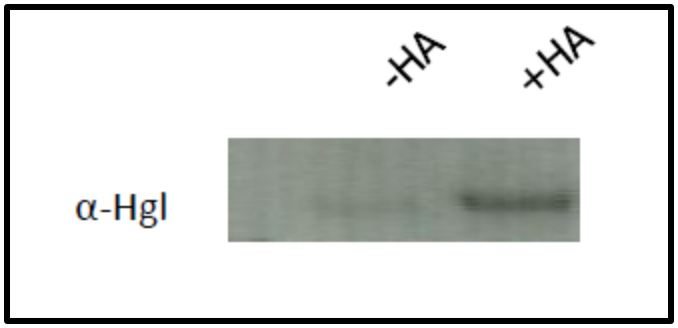

Figure 2. Acyl Biotin Exchange shows palmitoylation of Hgl in wildtype cells. Cells (1 x 10^7) were lysed and subjected to the ABE protocol (43). Western blot of samples (± hydroxyl amine or HA) was performed for Hgl in order to determine the palmitoylation status.

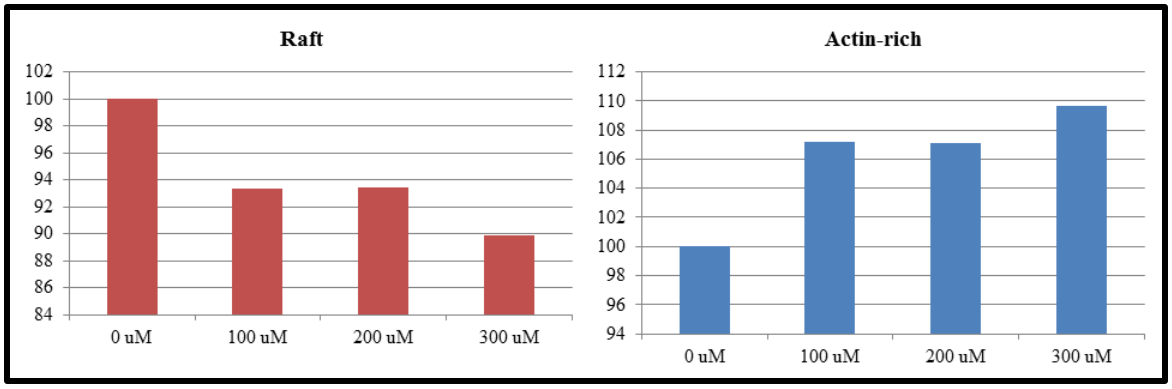

Figure 3. Chemical disruption of palmitoylation inhibits association of Hgl with raft-fractions. Trophozoites were exposed to 100 µM 2-bromopalmitate or an equivalent volume of ethanol (diluent control) for 2 hours prior to raft extraction. Fractions were pooled as raft (9-12) or actin-rich (17-20P) and subjected to Western blotting and densitometry. The data represent the densitometirc units of 1 trial and are reported as percent of DMSO control which was arbitrarily set to 100%.

Inhibition of GPI-anchoring is associated with Hgl enrichment in raft fractions

Lgl and Igl both have GPI-anchored forms (15). It is currently unknown what role, if any, that GPI-anchors have in regulating the submembrane distribution of Lgl and Igl. Therefore, we used both biochemical and genetic approaches to assess this role. We chemically inhibited GPI-anchoring using the serine esterase inhibitor, phenylmethanesulfonylfluoride (PMSF), which has been used to inhibit GPI synthesis in live *Trypanosoma brucei* (31). PMSF works by inhibiting the incorporation of phosphoethanolamine into the GPI precursor; importantly, it does not affect *N*-glycosylation (31). The amount of GPI-anchoring in cells was measured using fluorescently labeled aerolysin (FLAER) staining. The maximal dosage of 0.5 mM PMSF that did not affect cell viability resulted in 30% inhibition of FLAER staining (Figure 4). There was a slight enrichment of Lgl in rafts, but no noticeable change in the submembrane localization of Igl after PMSF treatment (Figure 5). Interestingly, PMSF exposure resulted in an enrichment of Hgl in lipid rafts (Figure 5).

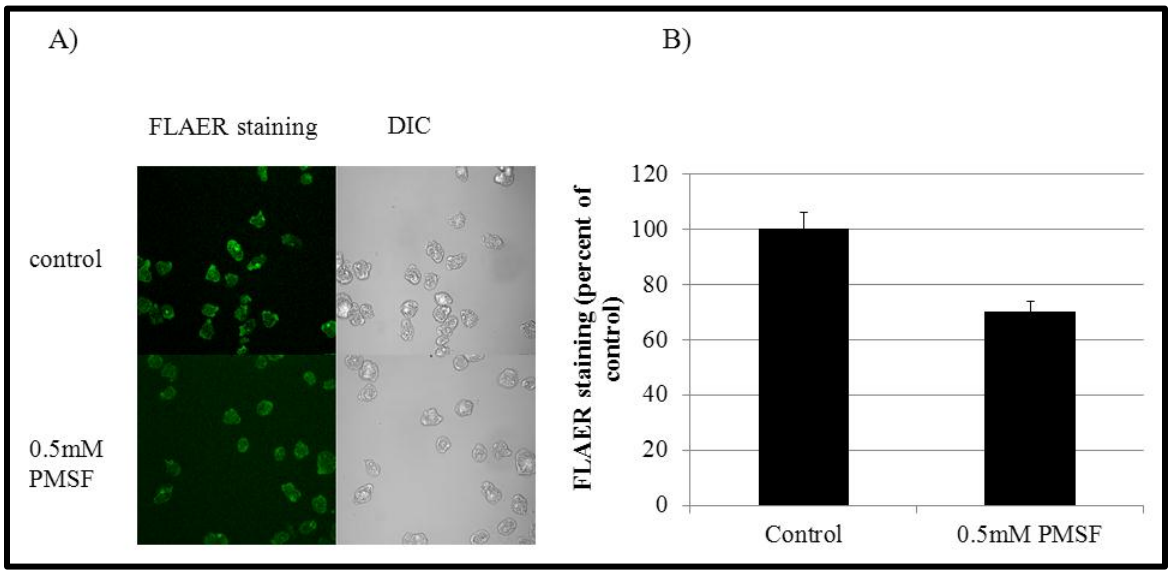

Figure 4. Exposure to PMSF reduces GPI-anchoring as measured by fluorescently labeled aerolysin (FLAER) staining. Cells were exposed to 0.5 mM PMSF for 1 hour prior to staining A) Cells were stained with FLAER and B) FLAER staining was quantified by measuring fluorescence/area (n=3 average ± S.D.).

To confirm that the effect of PMSF on Hgl was not due to off-target effects of the chemical, or of its diluent, we used a genetic model. We expressed an antisense Eh-PIG-M1 construct, which targets the phosphatidylinositol glycan mannosyltransferase. Expression of antisense Eh-PIG-M1 induces a 4-5 fold reduction in GPI anchorage (as demonstrated by FLAER staining) (44). Previously, expression of this construct was shown to knockdown Eh-PIG-M1 protein levels by 60% (44). Genetic knockdown of GPI-synthesis in *E. histolytica* resulted in a loss of the majority of the Lgl-31 (GPI-anchored), but not Lgl-35, in buoyant fractions (9-12) (Figure 5). These data demonstrate efficacy and specificity of the anti-sense transcript since only the GPI-anchored Lgl was affected. As shown with PMSF treatment (Figure X), expression of the antisense version of Eh-PIG-M1 resulted in enrichment of Hgl in rafts (Figure 5) which suggested that interaction with a GPI-anchored protein may inhibit Hgl raft-association. The total level of Hgl did not noticeably change. Interestingly, the knockdown of GPI-anchoring did not noticeably affect the amount of Igl in either raft or actin-rich fractions (Figure 5), although there was a change in buoyancy of the Igl subunit after genetic knockdown (Figure 5). This suggests that Igl is not dependent on its GPI-anchor to be raft-associated.

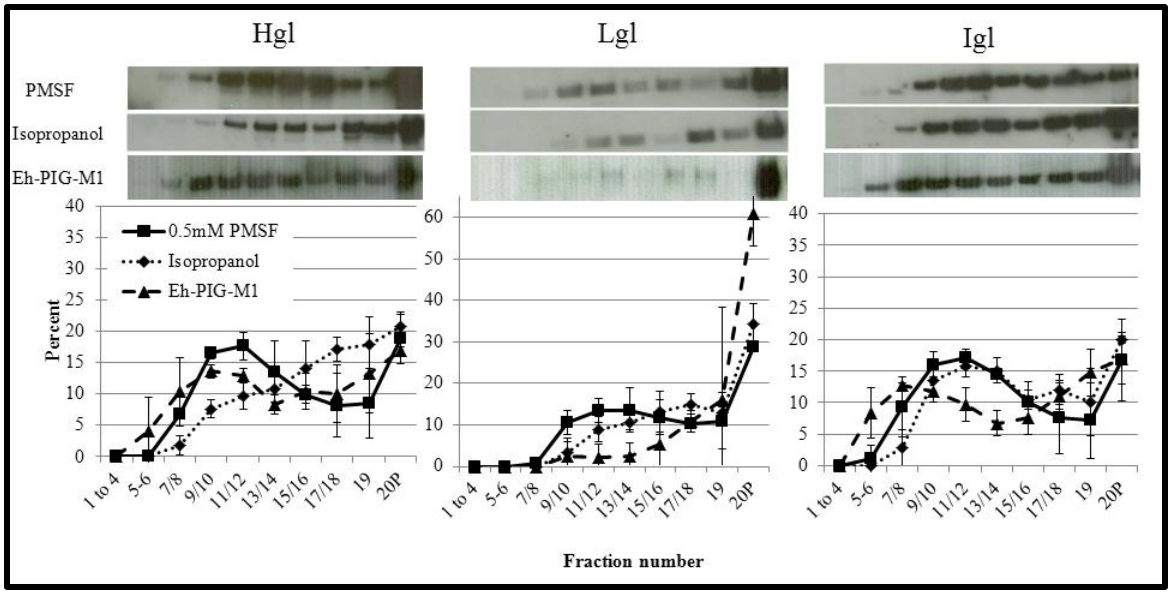

Figure 5. Chemical and genetic inhibition of GPI synthesis resulted in enrichment of Hgl in lipid rafts. Wildtype cells were exposed to 0.5 mM PMSF or isopropanol (diluent control) for 1 hour prior to raft-extraction. Antisense Eh-PIG-M1 expressing cells were also subjected to raft-extraction. After raft-extraction, sucrose gradient density fractionation was performed and fractions were collected. Western blots were performed on proteins isolated from fractions for A) Hgl, B) Lgl, and C) Igl. The densitometric analysis of western blots are reported as the percentage of total detergent resistant membrane per protein (n=2 average ± S.D.).

The role of the cytoplasmic tail of Hgl in lectin-raft interactions

Interactions between the cytoplasmic domain of a protein and other interacting proteins can also regulate raft association. Previously, a truncated version of HGL-2 was overexpressed, wherein the extracellular portion of HGL-2 was replaced with a FLAG epitope (HGL-2) (40). Expression of this construct resulted in reduced adhesion to enterocytes (40). We first wanted to determine if truncated HGL-2, which was missing its extracellular domain, was still able to interact with detergent resistant membrane. The truncated version of Hgl, itself (HGL-2) was localized to actin-rich DRM fractions (Figure 6). We next determined what effect the expression of the HGL-2 construct would have on localization of endogenous Hgl, Lgl, and Igl. Endogenous Hgl was enriched in lipid rafts in HGL-2-expressing mutant cells as compared to vector control (Figure 6). There were slight changes to the localization of Lgl, including increased amount of Lgl in fractions 11-12 (Figure 6). Igl localization remained unchanged in HGL-2 expressing cells (Figure 6).

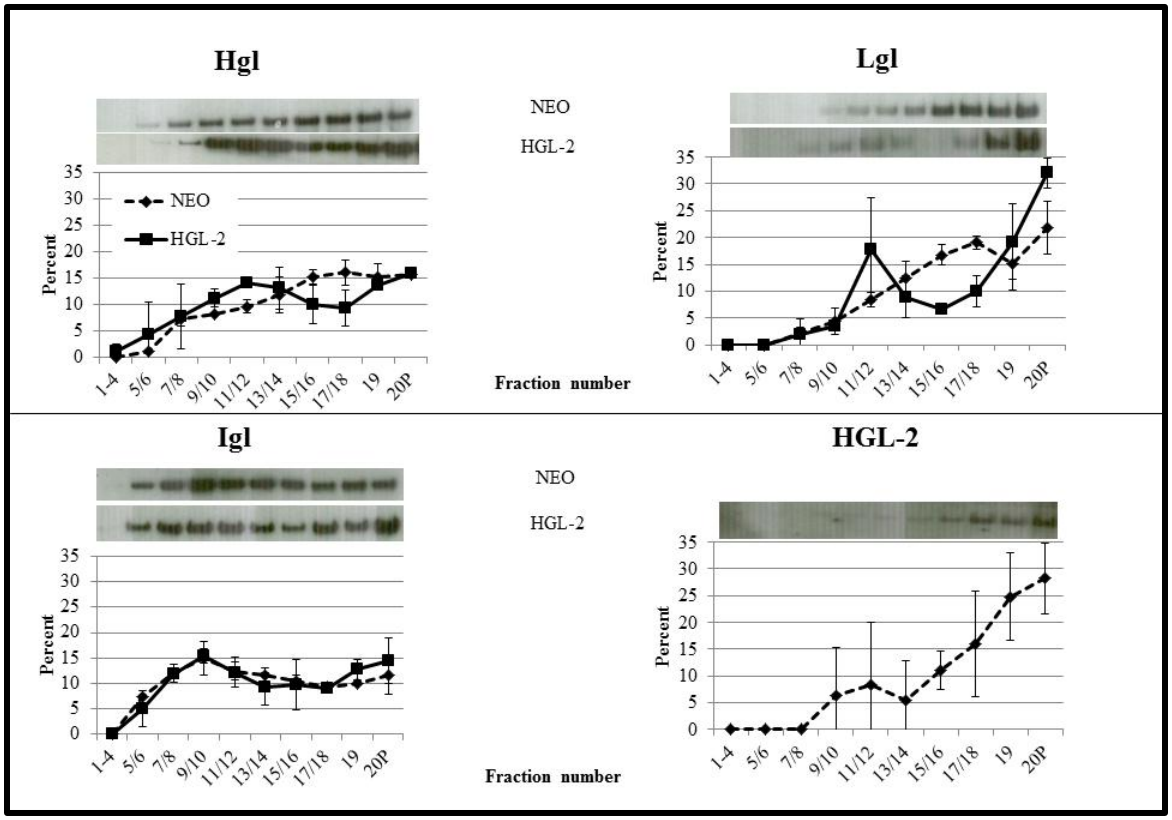

Figure 6. Expression of HGL-2 is associated with enrichment of endogenous Hgl in rafts. Hgl extracellular-truncation expressing cells, HGL-2, or empty vector control, NEO, were subjected to raft-isolation, sucrose gradient density fractionation, and fraction collection. Western blots were performed on proteins isolated from fractions for A) Hgl, B) Lgl, C) Igl, and D) HGL-2 cytoplasmic truncation. The data represent the average and standard deviations for densitometric scans of western blots which are reported as the percentage of total detergent resistant membrane per protein (n=2).

Overexpression of the transmembrane and cytoplasmic domain of Hgl (HGL-2) induced enrichment of endogenous Hgl in rafts. One explanation for this is that proteins that interact with the cytoplasmic domain of Hgl maintain its non-raft-association in steady state. The truncated protein titrated these interactors thus releasing endogenous Hgl to interact with rafts. The cytoplasmic domain of Hgl has been previously shown to interact with a number of proteins, including the cytoskeletal proteins actin, α-actinin, myosin, talin, and spectrin (18, 30, 32). Therefore, cytoskeletal elements were potential targets for proteins binding leading to the prevention of Hgl entering rafts. To determine if cytoskeletal elements were specifically interacting with the HGL-2 protein and not with endogenous Hgl, we performed immunoprecipitation of both the full-length and truncated Hgl and characterized the precipitated proteins by Western blots. As expected, FLAG-tagged HGL-2 was precipitated with the FLAG antibody, and the full length Hgl was precipitated with the monoclonal antibody to Hgl (Figure 7). Since the truncated HGL-2 protein no longer contains its extracellular domain, Lgl only interacted with the endogenous Hgl (Figure 7). We found that actin interacts with both endogenous Hgl as well as the truncated HGL-2 (Figure 7). Therefore, at this time we have not determined which protein interacts with the cytoplasmic domain of Hgl to prevent its association with lipid rafts.

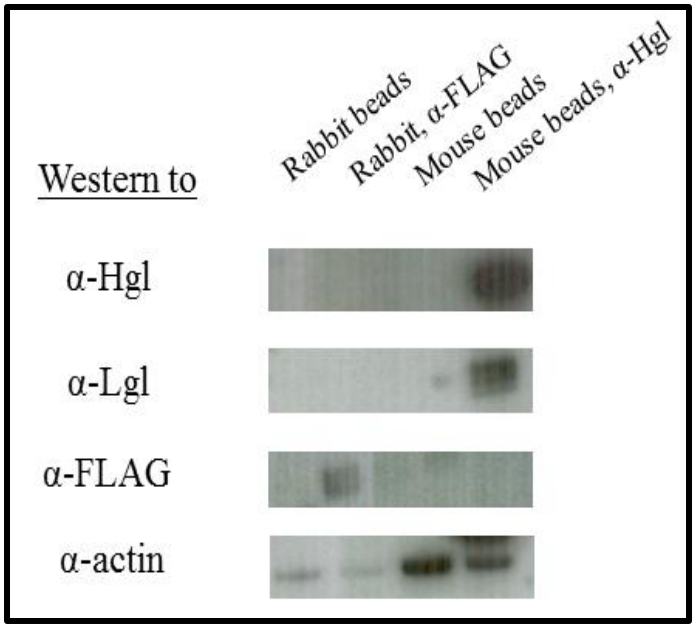

Figure 7. Immunoprecipitations on cells expressing HGL-2 construct do not show a difference in interaction between endogenous Hgl or HGL-2 protein with actin protein. HGL-2 cells (4×10^5) were incubated with either sheep anti-rabbit with or without FLAG antibody, or anti-mouse dynabeads with or without monoclonal Hgl antibody. SDS-PAGE and western blotting was performed on interacting proteins using antibodies specific for Hgl, Lgl, FLAG, and actin.

Depolymerization of actin is not sufficient to alter the buoyancy of Gal/GalNAc lectin subunits

A large body of evidence supports a role for a dynamic actin cytoskeleton in *E. histolytica*–host cell interactions (3-5, 25, 32). Therefore, we determined whether depolymerization of actin could affect the sub-membrane distribution of the lectin subunits. We employed CytoD, an actin depolymerizing agent, to reduce the level of F-actin in trophozoites. FITC–phalloidin-staining and fluorescence microscopy of CytoD-treated trophozoites revealed a substantial loss of polymerized actin, supporting the utility of this reagent in this system (appendix Figure A-1). DRM was isolated and characterized from control and CytoD-treated amoebae. After treatment with CytoD, actin was minimally detected in buoyant fractions (appendix A-2), further supporting the ability of CytoD to disrupt cytoskeletal-membrane interactions in *E. histolytica*. However, CytoD-treatment, and thus loss of polymerized actin, failed to induce redistribution of any of the lectin subunits (Appendix A-2). Therefore, simple loss of actin is not sufficient to permit the association of Hgl and Lgl with lipid rafts or the loss of Igl from lipid rafts.

Discussion

In this study, we have explored the role of posttranslational modifications, the cytoplasmic tail domain of Hgl, and actin in the localization of Gal/GalNAc lectin subunits in rafts. We have found that palmitoylation and glycosylation appear to be positive regulators of Gal/GalNAc lectin-raft interaction while GPI-anchoring and the cytoplasmic tail of Hgl appear to be negative regulators of this association.

In mammalian epithelial cells, glycosylation has been shown to be an apical sorting signal and a raft-association signal. In studies where both non-glycosylated and glycosylated versions of a protein exist, differences in glycosylation status regulate raft association. For example, only the glycosylated form of CNGA2 (subunit of the olfactory CNG channel) is raft-associated (6). There is a preference for rafts for the glycosylated forms of UT-A1 urea transporter and rat mu opioid receptor, although non-glycosylated forms are found in smaller amounts in rafts (8). Epidermal growth factor receptors are recruited to lipid rafts by N-linked glycosylation sites in their extracellular domain (47). Therefore, it was not surprising that tunicamycin exposure led to the disassociation of a highly glycosylated Lgl with lipid rafts. It is interesting that only Lgl-31 remained in rafts after tunicamycin treatment. This suggests that glycosylation is only important for the association of the non-GPI anchored Lgl with rafts. Since, the total amount of Lgl in actin-rich fractions did not decrease, we cannot rule out the possibility that Lgl-35 is still raft associated but simply smaller due to due to the removal of glycan groups.

Glycosylation may prove to be the most important raft-targeting signal for Lgl-35 because this version has no GPI anchor. This isoform of Lgl has been shown to regulate pathogenicity. Antisense inhibition of the Lgl-35 inhibits cytoxicity and cytopathogenecity, but not adhesion to baby hamster kidney (BHK) cells (2). Lgl1, which is one of two genes encoding the 35 kDa subunit, is transcriptionally dominant, making up 85% of the Lgl transcript found in *E. histolytica* trophozoites (2, 19). Additionally, this isoform of Lgl was underrepresented in the nonvirulent Rahman strain

(2). To date, the precise role of GPI-anchors in lipid raft association of *E. histolytica* has not been elucidated.

Although it was shown that Hgl is palmitoylated and that 2-bromopalmitate-induced removal of palmitoyl groups affected the raft localization of Hgl, we cannot conclude that that palmitoylation on Hgl, itself, is responsible for raft or non-raft association of Hgl. Therefore, further studies, such as mutation of predicted palmitoylation sites on Hgl, will need to be performed to determine whether non-palmitoylated Hgl is able to associate with rafts. Palmitoylation has been previously shown to be an important raft targeting signal, in both mammalian cells and *Trypanosoma* species. In trypanosomes palmitoylation is responsible for the targeting of calcium binding proteins to flagellar raft membrane (29). Inhibition of palmitoylation in *Toxoplasma gondii* inhibits essential processes such as invasion of host cells and motility (1). Since the Gal/GalNAc lectin has been shown to be an important virulence factor, the palmitoylation of Hgl (this study, Petri paper), may also have similar effects on overall virulence in *E. histolytica*.

GPI-anchored proteins have long been identified as raft-constituents. Interestingly, there are examples of GPI-anchored proteins that do not associate with rafts. In *E. histolytica*, GPI-anchored Igl is always a raft-constituent, while the GPI-anchored Lgl is not. Unexpectedly, both chemical and genetic knockdown of GPI-anchoring resulted in enrichment of Hgl in rafts. This may indicate that the association of Hgl with a GPI-anchored protein (Lgl or other protein), inhibits its interaction with rafts in steady state. The genetic knockdown of GPI anchoring, leading to loss of Lgl-31 association with DRM, was not unexpected as GPI-anchors are often necessary for the localization of

proteins to lipid rafts. However, since the total level of Hgl did not change, it suggests that Hgl does not need to be associated with a GPI-anchored protein to localize to DRM. Others have shown that deletion of the GPI anchor cleavage/addition signal on Lgl, leads to its failure to associate with Hgl. (35). We observe an enrichment of Hgl in raft-fractions, and a complete removal of Lgl in rafts after genetic inhibition of GPI-anchors. This confirms the previous observation (35). Levels of Igl were not affected by either genetic or chemical knockdown of GPI synthesis in cells, suggesting that Igl does not o depend on a GPI anchor to be raft-associated. It is possible that the localization of Igl to both rafts and DRM is dependent on its association with other proteins; however, it is currently unknown which proteins may directly interact with Igl aside from the other lectin subunits.

The GPI anchors of *E. histolytica* have Gal-Man-Man-GlcN-myoinositol, which is a unique glycan backbone (41). Since Eh-PIG-M1 expression affects pathogenicity, adhesion, fluid-phase endocytosis, and ability to resist lysis by human serum, this may be due, in part, to the mislocalization (at the submembrane level) of proteins such as Lgl after GPI disruption.

Since overexpression of the cytoplasmic domain of Hgl caused enrichment of endogenous Hgl in rafts, we explored whether protein-interactions accounted for Hgl sequestering in actin-rich membrane. In T cells, integrins such as lymphocyte associated antigen 1 (LFA-1) and α4β1, are restricted from associating with rafts by the cytoskeleton; depolymerization of F-actin by treatment with CytoD permits their movement into these microdomains (22). On the other hand, depolymerization of actin in

myoblasts prevents the association of N-cadherin, another adhesion molecule, with detergent-resistant rafts (7). Therefore, actin can serve as a negative or positive regulator of protein–raft interaction in a cell-specific manner. In the current study, depolymerization of actin was not sufficient to increase the association of Hgl or Lgl with raft-like fractions. Thus, intact actin does not appear to behave as a negative regulator of Hgl or Lgl raft engagement in *E. histolytica*. Importantly, our data do not rule out the possibility that actin is a positive regulator lectin–membrane interactions. We also examined the association of other cytoskeletal proteins with endogenous Hgl and compared them to those that interact with the truncated HGL-2. However, no differences in protein interactions were seen that would indicate whether the interaction of the C-terminus of Hgl with other proteins retains the subunit in high density, non-raft actin-rich membrane during steady state.

This is the first study in *E. histolytica* to explore the mechanism by which proteins become enriched in lipid-raft domains. We have presented preliminary evidence that glycosylation and palmitoylation are positive regulators of protein-raft association, while GPI-anchoring and the cytoplasmic domain of Hgl are negative regulators of protein-raft association. This study is an important first step in elucidating the mechanisms regulating the association of the lectin subunits, as well as other proteins, with rafts in *E. histolytica*. Since both GPI-anchor synthesis and palmitoylation have been identified as potential drug targets in other protozoan parasites, understanding the role they play in *E. histolytica* could be important for designing future treatment options. Additionally,

understanding raft-biology in *E. histolytica* leads to a better understanding of virulence signaling pathways.

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CHAPTER 4

SUMMARY OF MAJOR RESEARCH FINDINGS

Entamoeba histolytica is a protozoan parasite, which is the causative agent of amebiasis and amebic liver abscess. During invasion, *E. histolytica* utilizes cell surface receptors, the best characterized of which is the Gal/GalNAc lectin (11). The Gal/GalNAc lectin is so named because it binds both galactose and N-acetylgalactosamine residues on host cells and ligands. The Gal/GalNAc lectin is comprised of three subunits, heavy (Hgl), light (Lgl), and intermediate (Igl). In steady state, Igl is localized to lipid rafts, or liquid ordered, cholesterol- and sphingolipid-rich membrane (4, 7, 15). The major questions addressed in this research are (1) how Hgl and Lgl subunits become raft-associated in a biologically relevant ligand-specific manner and (2) how posttranslational modifications and the C-terminal domain of Hgl regulate the localization of Hgl and Lgl in lipid rafts.

The review of the role of protozoan lipid rafts is an important resource, which can be used by numerous researchers who want to compare what is known in their system to others. Having all of this information in one document will undoubtedly spur new ideas across a number of parasitic species. For example, much of the lipid raft research in *Plasmodium falciparum* has carried out using proteomic approaches level, whereas much of the same research in *E. histolytica* has been accomplished using cell biological approaches. The review may provide the impetus to adopt techniques used for one parasite for the study of a different parasite. Although the focus of this dissertation

research was the role of rafts in adhesion and signaling, other studies described in this review outline additional roles for rafts in motility, secretion, and invasion of host cells.

Attachment to biologically relevant ligands

During infection, *E. histolytica* attaches to host cells, including human red blood cells (hRBCs) and extracellular matrix components (including collagen and fibronectin) (2, 5, 12). Attachment to hRBCs and collagen has been shown to be Gal/GalNac lectin-mediated interactions, whereas attachment to fibronectin has been shown to be lectin-independent (10). In mammalian cells, binding to ligand induces clustering of integrins in lipid raft domains (6).

I have shown that upon attachment to *bonafide* lectin ligands, such as hRBCs and collagen, there is an enrichment of the Hgl and Lgl subunits in lipid rafts. This enrichment required physical interaction between the lectin and these ligands. On the other hand, binding to fibronectin did not induce a similar enrichment of Hgl and Lgl in rafts. Igl remained localized in rafts in all of these conditions. To determine if binding to ligand was sufficient to induce enrichment of Hgl and Lgl in rafts, we used a cell line that expressed (GFP)-labeled PH domain derived from Brutons Tyrosine Kinase (PH^{BTK}), GFP-PH^{BTK}. This cell line was previously shown to bind to but not phagocytose hRBCs (1). Exposure of this cell line to hRBCs did not change the localization of Hgl or Lgl subunits in rafts indicating that binding was not sufficient to alter the submembrane localization of these subunits.

This study was the first to show that Gal/GalNAc mediated parasite-host interactions modulate the submembrane localization of this adhesin. Since the Gal/GalNAc lectin is critical for virulence, the data provide insight into pathogenicity. In the future, it will be necessary to determine what changes might occur in the subunits of the GalGalNAc lectin after ligand binding that alters their affinity for rafts. For example, in there may be a conformational change in the lectin subunits which may change their localization from actin-rich membrane to lipid raft membrane. Atomic force microscopy of the surface of amoebae may provide such insight.

Calcium and PIP_2 are required for enrichment of Hgl and Lgl in rafts to occur

We hypothesized that the GFP-PH^{BTK} cell line might have altered phosphoinositide signaling. Therefore, we measured levels of PIP_2 in this cell line versus wildtype and we found that PIP_2 levels were decreased in the GFP-PH^{BTK} cell line compared to wildtype levels. We then demonstrated that PIP_2 loading could partially rescue the enrichment of Hgl and Lgl in lipid raft domains, indicating that sufficient PIP_2 levels are essential for enrichment to occur.

Since hydrolysis of PIP_2 leads to the release of intracellular calcium stores, we determined if intracellular calcium levels increased in wildtype cells upon binding of collagen. We found a statistically significant increase in calcium upon binding of collagen, ascompared to binding of fibronectin. Finally, we used calcium-chelation with BAPTA-AM to determine if increased levels of calcium were necessary for the enrichment of lectin subunits in rafts to occur. Binding of ligand in the presence of

BAPTA-AM did not induce a change in submembrane localization of lectin subunits, indicating a role for intracellular calcium in raft-localization. BAPTA-AM did not affect the actual binding of cells to collagen, confirming that changes in adhesion were not responsible for the lost enrichment phenotype.

This research was significant for a number of reasons. First, we have shown that PIP_2 loading is a useful tool in *E. histolytica*. Therefore, this could be useful for future research as there are a number of phosphoinositide species that can be loaded in this manner and have yet to be studied in *E. histolytica*. Second, we are the first group to indicate, in any system, a role for PIP_2 in raft-localization. This may translate into a number of systems. Third, this study gives us insight into the signaling pathway that occurs during enrichment of lectin subunits in rafts. In the future, it will be necessary to identify effectors that act downstream and in parallel with the Gal/GalNAc lectin after the enrichment of its subunits in rafts. Fully understanding how this receptor responds to extracellular ligands is necessary for understanding virulence functions in *E. histolytica.*

Role of glycosylation in raft localization

The role of posttranslational modifications in raft-association in *E. histolytica* has yet to be studied. Since Hgl and Lgl have potential N-linked glycosylation sites, we used chemical removal of N-linked glycans to determine if this posttranslational modification modulates the localization of these subunits. It is important to point out that although both 31 kDa and 35 kDa Lgl are predicted to be glycosylated, the 31 kDa subunit has fewer potential glycosylation sites and is also GPI anchored (9). Tunicamycin treatment,

which effectively blocks N-linked glycosylation in *E. histolytica* (11), decreased levels of both Hgl and Lgl (35 kDa isoform) in raft-fractions. There was no change in the total amount of Hgl or Lgl in actin-rich fractions, although there did not appear to be a band at 35 kDa (glycosylated Lgl), which may indicate that the subunit has changed in size due to the loss of glycan groups. Hgl changed in size, as was previously published (9).

The role of glycosylation in raft localization has been explored in mammalian systems. Although chemical knockdown of glycosylation is not ideal, our results do indicate a role for glycosylation in raft-localization of Hgl and 35-kDa Lgl. Future studies using site specific alterations of predicted glycosylation sites will reveal that glycosylation of Hgl or Lgl regulate raft-association. It would also be necessary to determine if Hgl and 35-kDa Lgl are still physically bound to each other after reducing N-glycosylation. Loss of association between the subunits may be significant as it is currently unknown which subunit directs the other into rafts.

Role of palmitoylation in raft localization

Palmitoylation is the most common posttranslational modification of raft-associated proteins in mammalian cells (8). It is also important for raft-association in at least one other protozoan parasite, *Trypanosoma* spp (3). We used acyl biotin exchange (ABE), which effectively converts palmitoyl groups to biotin in order to extract palmitoylated proteins, to confirm that Hgl is palmitoylated. We also used a chemical inhibitor of palmitoylation, 2-bromopalmitate, to show that chemical knockdown of palmitoylation

was associated with loss of Hgl in raft-fractions, and gain of Hgl in actin-rich non-raft fractions.

This work is important since knockdown of palmitoyl acyl transferases has been proposed as a possible therapeutic agent in trypanosomes. Only a handful of studies in *E. histolytica* have examined palmitoylation. This work also demonstrates the utility of the ABE method for studying palmitoylation in *E. histolytica.* In the future, ABE may be used to define the entire raft and non-raft palmitoyl proteome of *E. histolytica*.

Role of GPI-anchors in raft localization

Both Igl and the 31-kDa Lgl are GPI-anchored. We used both biochemical and genetic approaches to determine if GPI-anchoring was important for raft-association. Biochemical treatment with PMSF, which prevents the incorporation of phosphoethanolamine into the GPI precursor, produced a GPI-knockdown of 30% as measured by FLAER staining. In PMSF treated cells, Hgl became enriched in lipid rafts. However, negligible changes in submembrane location were observed for Lgl and Igl. Expression of an antisense construct, Eh-PIG-M1, which was previously shown to knockdown GPI-anchoring by 4-5 fold (14), was also used in order to determine the role of GPI-anchoring. Expression of antisense Eh-PIG-M1 was also associated with enrichment of Hgl in rafts and a a near complete loss of buoyant 31 kDa Lgl. Interestingly, it did not affect the localization of Igl.

One explanation for the enrichment of Hgl in rafts after genetic or biochemical inhibition of GPI-anchoring is that Hgl interacts with a GPI-anchored protein (Lgl or

other protein) that prevents its association with rafts. Genetic inhibition of GPI-anchoring demonstrated that the 31-kDa Lgl is dependent on GPI-anchoring for its localization to buoyant membranes including rafts. Genetic and chemical data also show that Igl may not depend on its GPI-anchor for raft association. It is possible that Igl is ferried into and maintained in rafts through interactions with other proteins. Future studies should be carried out to determine what proteins interact with Hgl, Lgl and, Igl which may provide insight into the mechanisms regulating protein-raft intereactions in this parasite It is important to also note that GPI-synthesis has been proposed as a potential drug target in both *E. histolytica* and other protozoan parasites. A better understanding of the role of these lipid anchors will be necessary to pursue such avenues of drug design.

Hgl Cytoplasmic Domain

Interactions between the cytoplasmic domain of a protein and other proteins can regulate raft association. A truncated version of Hgl, HGL-2, in which the extracellular portion of HGL-2 was replaced with a FLAG epitope (13) was overexpressed. While the HGL-2 protein was only localized to actin-rich membrane, endogenous Hgl was enriched in lipid rafts in the transgenic cell line indicating a role for the cytoplasmic domain of Hgl in raft-association. We used immunoprecipitation with anti-FLAG or anti-Hgl antibodies to try to identify proteins that may be interacting with Hgl or HGL-2. Our studies did not uncover any proteins that uniquely associate with the endogenous full-length or exogenous truncated Hgl subunit. More extensive proteomic studies may reveal such

interacting proteins. Since Hgl interacts with actin, we specifically asked if actin could be acting as a negative regulator of lectin localization in rafts. We depolymerized actin using cytochalasin D and characterized the submembrane postion of Hgl. Complete depolymerization of actin did not lead to enrichment of Hgl or Lgl in rafts suggesting that actin is not a negative-regulator of Hgl-raft interaction. However, it remains to be determined if actin is a positive regulator of lectin-raft association.

Overall Impact of These Studies

The studies completed for this dissertation have added to the ever-growing field of lipid raft research. Since most studies of rafts have been carried out using mammalian cells, our studies in a lower eukaryote provide unique perspective. Although we find similarities between our system and mammalian cells (ligand binding studies, palmitoylation) we also have added new knowledge that has not previously been shown in other systems (PIP_2 and calcium regulation of raft-associations. Therefore, we have not only contributed to the field of research concerned with *Entamoeba* research but to the field of study concerned with rafts as a whole. Understanding mechanisms by which raft-association occurs in *E. histolytica* contributes to our understanding of the virulence of the parasite.

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APENDICES

Appendix A

Role of the actin cytoskeleton in raft localization of the Gal/GalNAc lectin

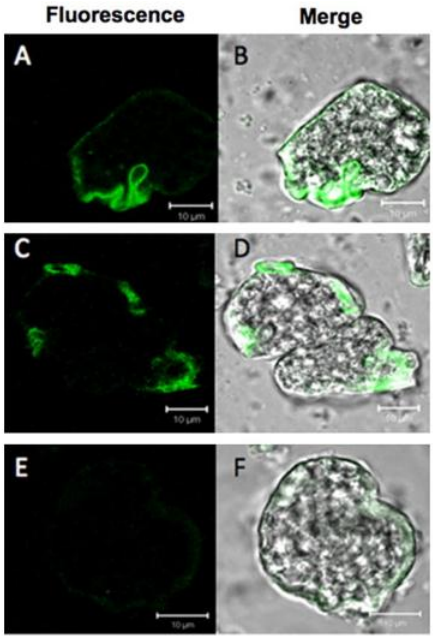

Figure A-1. Actin stain of cytochalasin D (CytoD)-treated *Entamoeba histolytica* trophozoites and untreated control trophozoites. Untreated control amoebae (A and B), amoebae exposed to CytoD diluent (DMSO) (C and D) or 10 μM CytoD (E and F) were stained with Alexa 488 (green)-conjugated phalloidin and visualized by confocal scanning fluorescence microscopy. Both fluorescence (A, C and E) and merged differential interference contrast (DIC) images (B, D and F) are shown. Actin is minimally detected in cells treated with CytoD, indicating that CytoD can induce the depolymerization of actin in *E. histolytica*. The scale bars represent 10 μm.

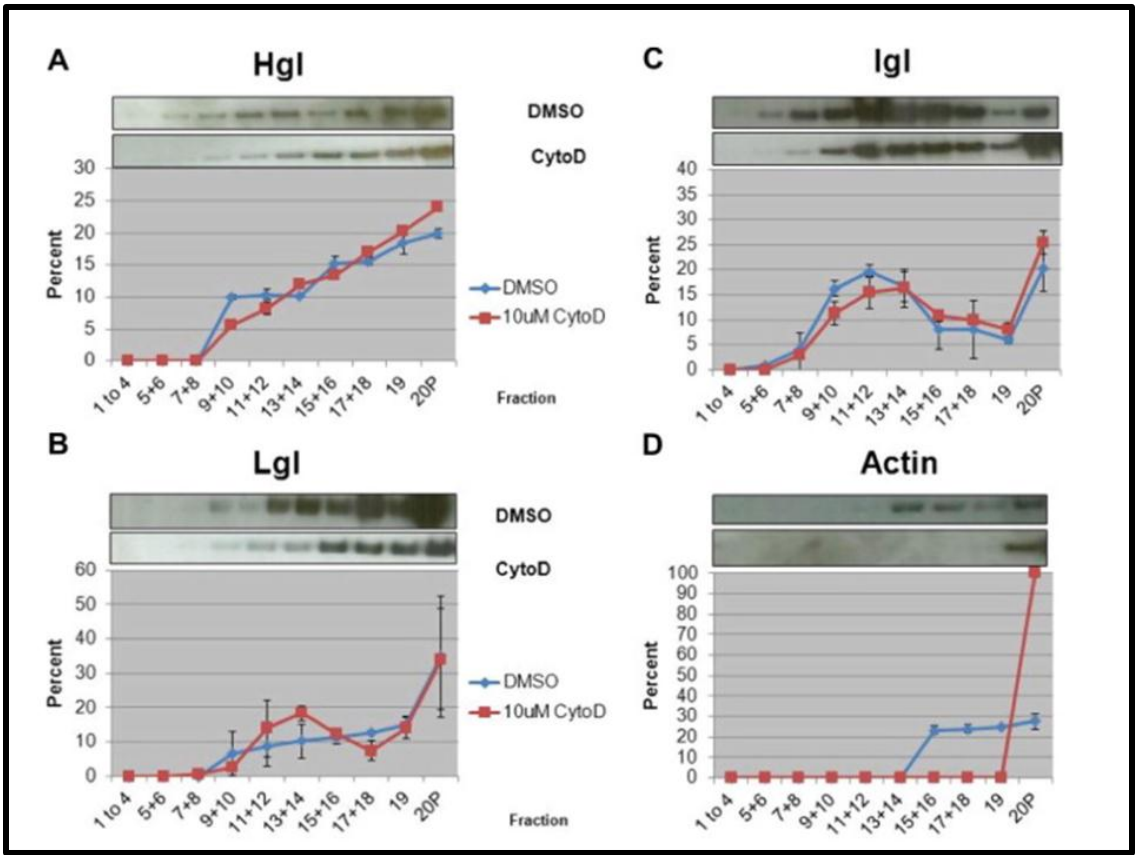

Figure A-2. Actin depolymerization has no effect on the sub-membrane distribution of the Gal/GalNAc lectin subunits. *E. histolytica* trophozoites were treated with 10 μM cytochalasin D (CytoD) or an equal volume of diluent control (DMSO). Triton-insoluble membranes were isolated and resolved by sucrose gradient centrifugation. Nineteen fractions and the pellet (20P) were collected and subjected to western blot analyses using antibodies specific for heavy (Hgl) (A), light (Lgl) (B), intermediate (Igl) (C) subunits or actin (D). Mean values of densitometric scans ($n = 2$), reported as a percentage of total detergent-resistant membrane (DRM)-associated protein, are shown for each subunit and actin. Representative western blots are shown above each panel. In treated cells, actin is only found in the non-buoyant pellet (D), indicating disruption of the cytoskeleton by CytoD. Hgl (A), Lgl (B) and Igl (C) exhibit identical sub-membrane distributions in control (blue line) and treated (red line) cells. International Journal of Parasitology, Vol. 41, Issues 13-14.

Data presented in Appendix A was published in the International Journal of Parasitology by Welter, BW, Goldston, AM, and Temesvari, LA entitled "Localisation to lipid rafts correlates with increased function of the Gal/GalNAc lectin in the human protozoan parasite, *Entamoeba histolytica*." This article was published in 2011 in volume 41, Issues 13-14, pages 1409-1419.

Appendix B

Effect of cholesterol on the actin cytoskeleton and adhesion

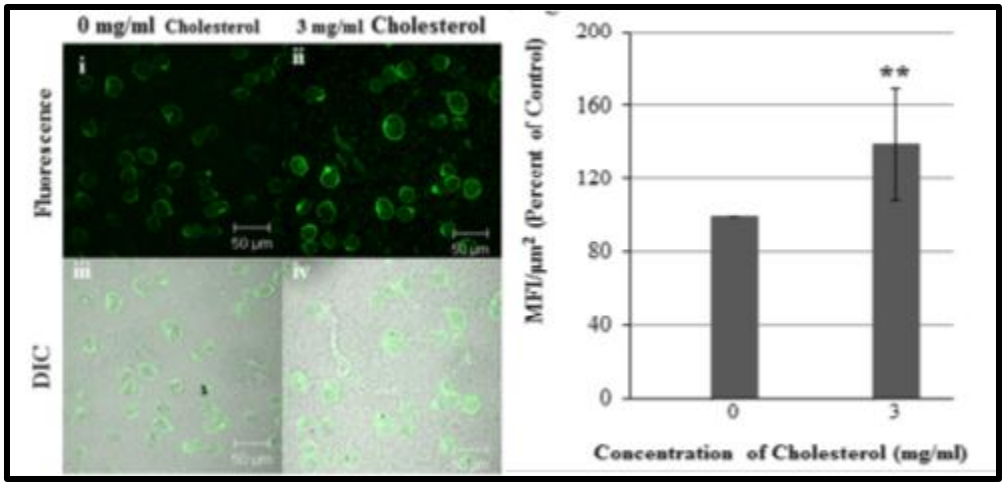

Figure B-1. Exposure to cholesterol enhances actin polymerization and adhesion in *E. histolytica* cells. Untreated control (0 mg/ml) and 3 mg/ml cholesterol-treated cells were stained with Alexa Fluor 488 (green)-conjugated phalloidin and visualized by confocal scanning fluorescence microscopy. (B, panel iii and iv) The corresponding DIC images are shown. Bars, 50 µm (C) Single confocal planes were used to measure the mean fluorescence intensity, MFI, of actin-staining using LSM510 Image analyzing software. Values were normalized for cell surface area. Cholesterol treatment significantly increased phalloidin-staining of whole cells suggesting that exposure to cholesterol resulted in increased polymerization of actin. The data are the means + S.D. from 3 independent experiments (**, $P<0.01$).

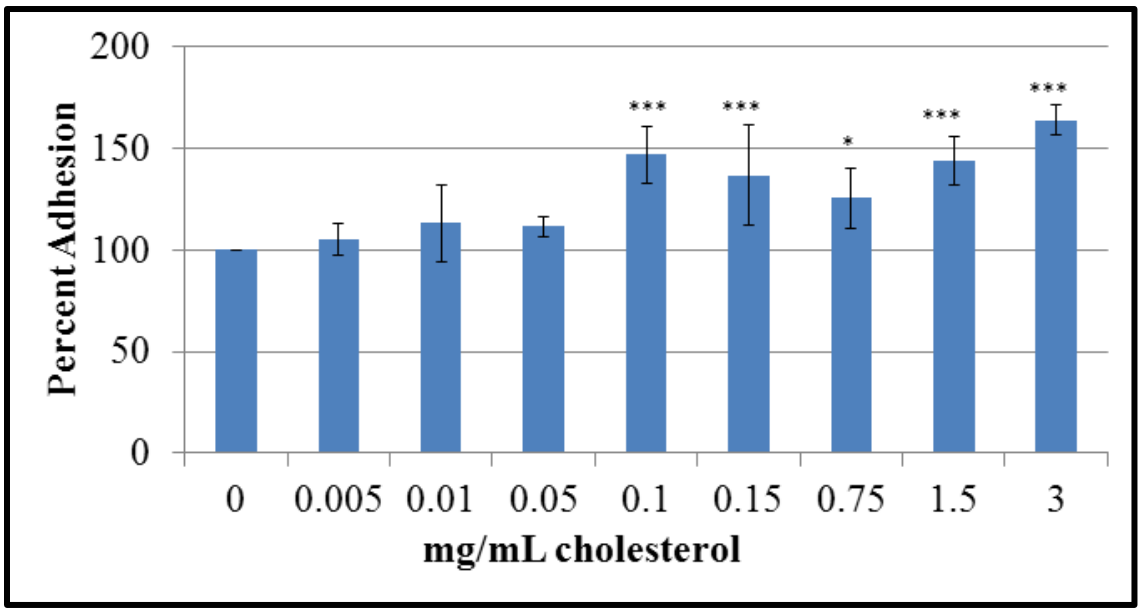

Figure B-2. Exposure to cholesterol increases adhesion to CHO cell monolayer. Trophozoites were preexposed to calcein-AM, then exposed to various concentrations of cholesterol. Following cholesterol incubation, cells were plated on CHO cell monolayer in 96 well plates and fluorescence was measured (485/530nm excitation/emission).

Data from appendix B is part of a paper which is currently under review by Koushik, AB, Powell, RR, Goldston, AM, and Temesvari, LA. This body of work used cholesterol as a tool to determine the role of PIP_2 in *Entamoeba histolytica* virulence. Specifically, the figures in this appendix show that cholesterol exposure increases phalloidin staining, which is a measure of F-actin within the cell. The figures also show that adhesion to CHO cells is also increased as a result of cholesterol treatment. The manuscript ties together PIP_2 signaling from lipid rafts with changes in actin, adhesion, and motility.

Appendix C

The effect of expressing Rab8CA and RacF2DN constructs on adhesion during development

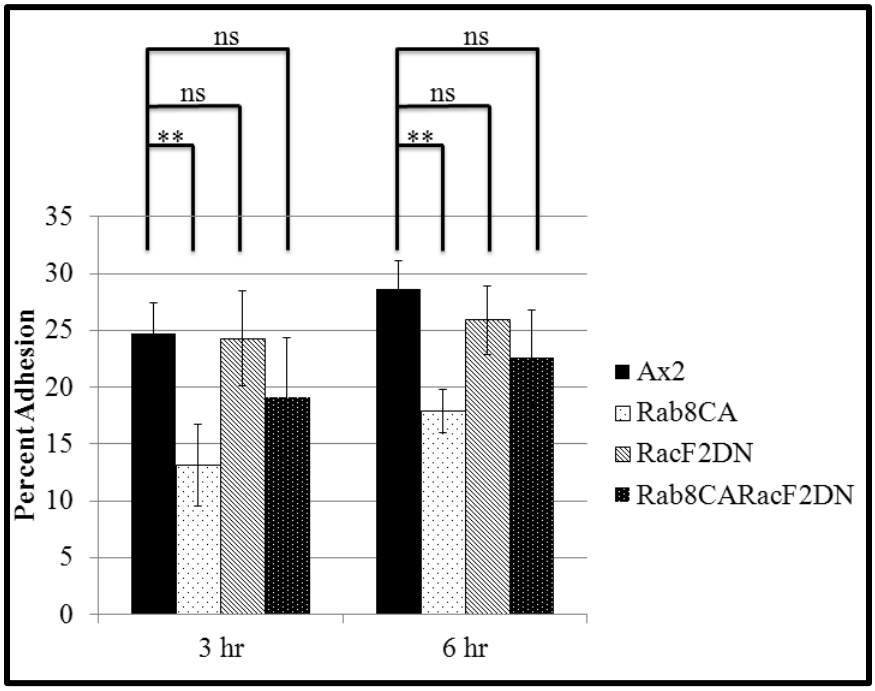

Figure C-1. Rab8CA expressing *Dictostelium discoideum* mutants exhibit significantly lower adhesion during development when compared to parental Ax2 cells. *D. discoideum* mutants were placed in starvation medium and allowed to develop in the dark for 3 or 6 hours. Following incubation, cells were vortexed briefly, and allowed to readhere to one another for 10 min. The number of cells adhered to one or more other cells was counted, and the percentage was calculated. Data was averaged ($n \geq 3$, +/- S.D.). (** $P < 0.01$). Rab8CA expressing cells exhibited lower adhesion during development, which could be rescued by the co-expression of RacF2DN.

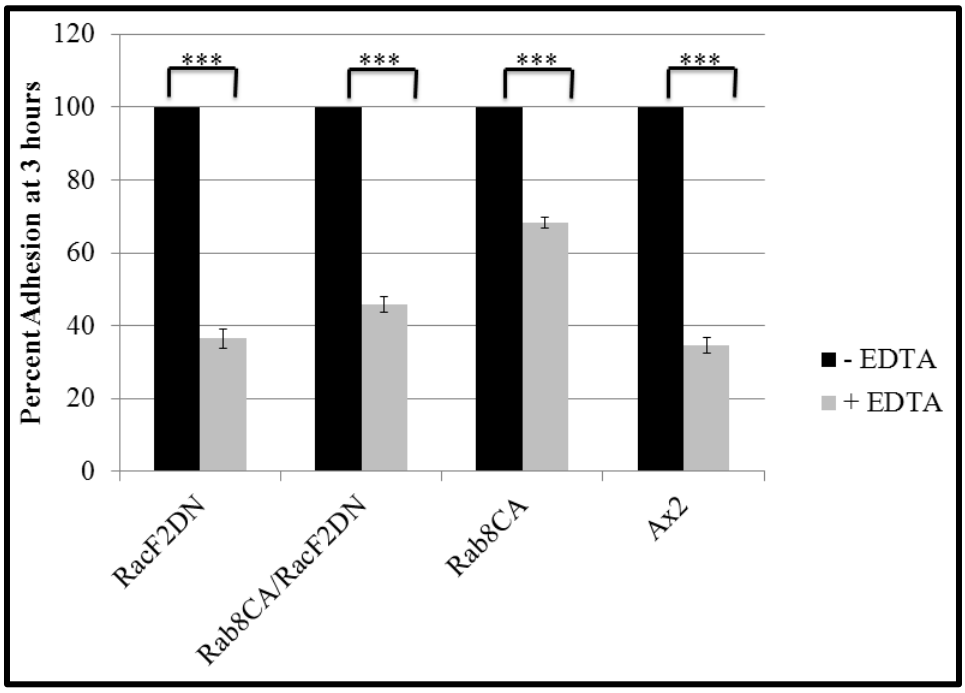

Figure C-2. *Dictostelium discoideum* mutants and parental cell lines exhibit EDTA-sensitive adhesion at 3 hour starvation. *D. discoideum* mutants were placed in starvation medium and allowed to develop in the dark for 3 hours. Following incubation, cells were vortexed briefly, and allowed to readhere to one another for 10 min in the presence or absence of EDTA. The number of cells adhered to one or more other cells was counted, and the percentage was calculated. Data was averaged ($n \geq 3$, +/- S.D.). (** $P < 0.01$). RacF2DN, Rab8CA/RacF2DN, and Rab8CA expressing cell lines all have significantly lower adhesion after calcium chelation by EDTA, however, Rab8CA appears to be less sensitive to EDTA treatment.

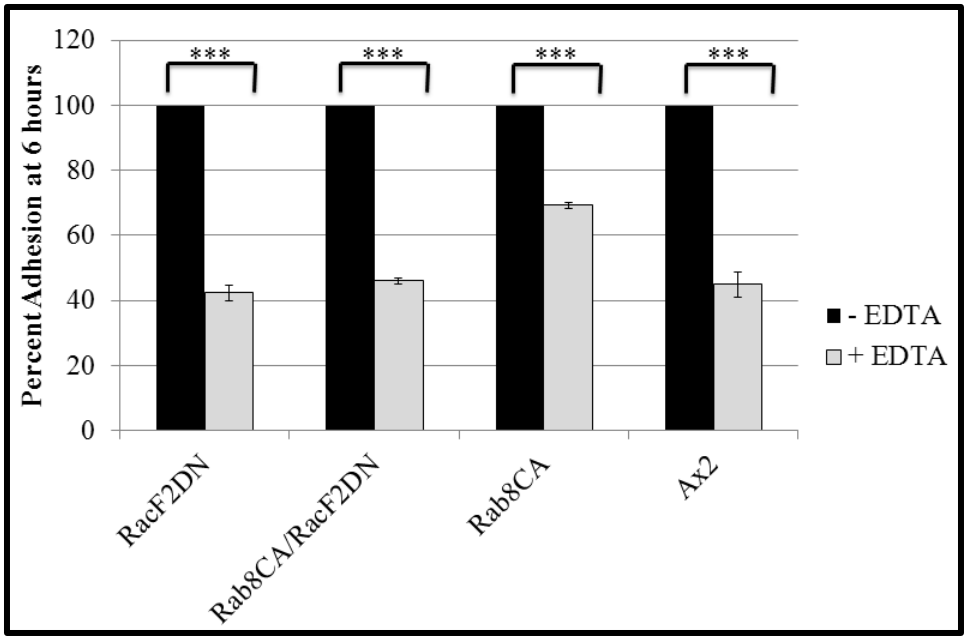

Figure C-3. *Dictostelium discoideum* mutants and parental cell lines exhibit EDTA-sensitive adhesion at 6 hour starvation. *D. discoideum* mutants were placed in starvation medium and allowed to develop in the dark for 3 hours. Following incubation, cells were vortexed briefly, and allowed to readhere to one another for 10 min in the presence or absence of EDTA. The number of cells adhered to one or more other cells was counted, and the percentage was calculated. Data was averaged ($n \geq 3$, +/- S.D.). (** $P < 0.01$). RacF2DN, Rab8CA/RacF2DN, and Rab8CA expressing cell lines all have significantly lower adhesion after calcium chelation by EDTA, however, Rab8CA appears to be less sensitive to EDTA treatment.

Data presented in Appendix C is part of a paper which is currently in preparation on the role of RacF2 and Rab8 on development of *Dictostelium discoideum*. The data presented show that expression of Rab8CA (Rab8 constitutively active) causes a significant decrease in the adhesion of *D. discoideum* cells during starvation. It also demonstrates that both RacF2DN (dominant negative) can rescue the decreased adhesion exhibited by Rab8CA cells. Furthermore, the data suggests that adhesion is EDTA-sensitive in all mutants: however, Rab8CA is less affected by EDTA than RacF2DN or double mutants (Rab8CA/RacF2DN) cells.

Appendix D

Cross reaction data for antibodies used in these studies against hRBC proteins

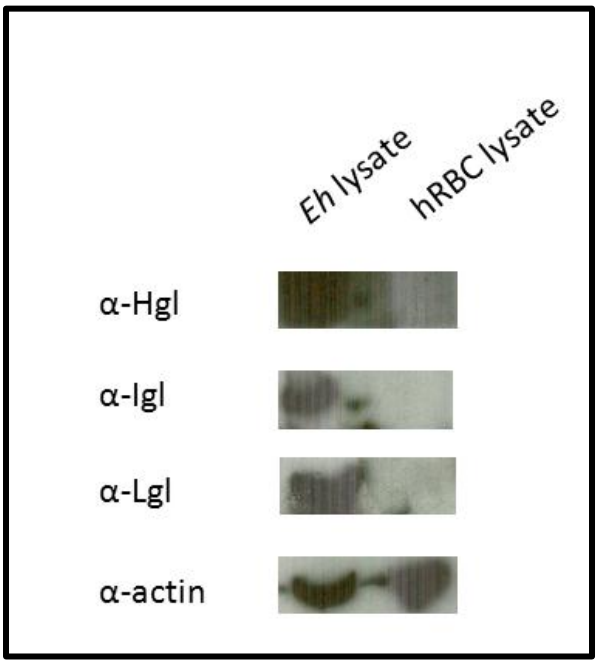

Figure D-1. Lectin antibodies only react with *E. histolytica* (*Eh*) lysate, and not red blood cell (hRBC) lysate. Trophozoites and hRBCs were lysed and western blot analysis was performed on cell lysate. Antibodies to the Gal/GalNAc lectin subunits (Hgl, Lgl, and Igl) and actin were used to determine if cross reaction between hRBC proteins and the antibodies occurred. Only α-actin antibody reacts with both *E. histolytica* and hRBC proteins.

Data presented in appendix D represents data which was not shown in the published version of chapter 2 (appeared as data not shown). It was performed as a control to determine if cross reaction would occur with proteins from red blood cells (hRBCs), in order to make sure no cross reaction occurred between the lectin antibodies and hRBCs during trophozoite exposure to hRBCs.